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ON THE BEHAVIOR OF BUFO AND RANA TOWARD COLLOIDAL DYES OF THE ACID AZO GROUP (TRYPAN BLUE AND DYE NO. 161)

. WITH REFERENCE TO THE PORTAL OF ENTRY OF THE DYE, AND THE CAUSES WHICH UNDERLIE THE INITIATION OF THE PROCESS BY WHICH COLLOIDAL DYE PARTICLES ARE STORED IN THE CYTOPLASM OF CERTAIN TYPICAL CELLS OF THE EMBRYO, AND
II. WITH REFERENCE TO THE DEVELOPMENT OF THE LYMPHATIC SYSTEM

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ON THE BEHAVIOR OF BUFO AND RANA TOWARD COLLOIDAL DYES OF THE ACID AZO GROUP (TRYPAN BLUE AND DYE NO. 161)

I. WITH REFERENCE TO THE PORTAL OF ENTRY OF THE DYE, AND THE CAUSES WHICH UNDERLIE THE INITIATION OF THE PROCESS BY WHICH COLLOIDAL DYE PARTICLES ARE STORED IN THE CYTOPLASM OF CERTAIN TYPICAL CELLS OF THE EMBRYO, AND II. WITH REFERENCE TO THE DEVELOPMENT OF THE LYMPHATIC SYSTEM

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1. INTRODUCTION

A study of the reactions of living tissues toward colloidal acid dyes has within recent years claimed the attention of a good many investigators. Investigations have thus far of necessity, however, been confined, for the most part, to a study of tissues in adult animals. Evans and Schulemann ('14) have advanced a strong argument in favor of the view that vital staining of certain tissues by colloidal acid dyes is purely a physical process and primarily one of ingestion or phagocytosis. This process consists in the ingestion by the cell of ultramicroscopic colloidal particles of dye which become stored in its cytoplasm in the visible form of what are now commonly designated as dye granules.

Although there appears to be little if any doubt that colloidal dye particles are taken up by certain types of cells by a process of phagocytosis, there may still be some doubt whether all of the cells which have thus far been observed to react toward these dyes may do so in exactly this same manner. While fully appreciating that the method by which dye gains entrance to the cell and that the significance of the reactions observed may not be the same in all cases, the nature of this investigation which deals chiefly with the portal of entry of the dye and its transportation, has demanded, in considering their behavior, that all tissues which react toward the dye should be considered more or less in common (Chapter 11).

A comparison of the tissues which react toward colloidal acid dyes, as thus far observed in vertebrates, shows that they are for the most part derived from embryonic mesenchyme and mesothelium. Cells derived from embryonic entoderm and ectoderm have also been observed to react toward these dyes.

The derivatives of mesenchyme which react toward the dye are largely confined to the vascular system. They include its venous and lymphatic endothelium to a greater or less degree and some, at least, and possibly all of the so-called white blood-corpuscles. Apart from the vascular system the derivatives of mesenchyme which have been observed to react toward the dye include cells of the reticulum in certain organs, fibroblasts, the interstitial cells of the testis, granulosa cells of the atretic follicle (Evans, '16) connective tissue cells and giant cells of the placenta.

By far the most important cells of mesothelial origin which react toward acid dyes are the epithelial cells of the renal tubules. Cells of mesothelial origin which have also been observed to react toward the dye are those which line the walls of the coelomic cavity and cells of the epicardium and adrenal bodies.

The ectodermal cells of the placenta have been observed to react toward the dye; also cells of entodermal origin, as the epithelium of the pharynx (anura), hepatic cells and cells of the epithelial bodies.

Although it is evident that the storage of colloidal acid dye particles is not accomplished in all of the above-mentioned cases by a process of phagocytosis, the circumstance that the reactions toward acid dyes are limited and confined exclusively to the cells of certain typical tissues appears to be a question of considerable significance. What these reactions may consist of in individual cases is not especially concerned in the present investigation.

Wilsocki ('16, '16a) has recently observed when young frog larvae are placed in a solution of colloidal acid dye, as trypan blue, that the latter gains admission to the body and certain tissues, including lymphatic endothelium, then react toward the dye in a typical and characteristic manner. The circumstance that dye gets into the circulation in some manner and so eliminates the necessity of injecting it into such young larvae, has somewhat broadened the possibilities of the field of inquiry. It may enable us to determine the exact time at which the macrophages and other tissues of the embryo first begin to react toward the dye, as well as the causes which underlie the initiation of such reactions. We may also be enabled to study the progressive reactions of the tissues toward these dyes during the different stages of larval development. It is possibly not quite clear what the exact source of origin of all the different types of white blood-corpuscles may be that are found in the embryonic body. As the endothelium of the lymphatic system, and, to a considerable extent, also that of the venous system has been found to be vitally stained by these dyes in young toad larvae, it may now be possible to distinguish more clearly, than has hitherto been the case, between the white blood-cells of strictly endothelial origin and those which arise directly from mesenchyme. The portal of entry and transportation of the dye in larval and adult amphibia, as well as the reactions of the tissues toward colloidal acid dyes during the period of hibernation, are also among the questions which have suggested themselves as worthy of further inquiry.

The present investigation was begun chiefly with the intention of examining the claim made by Wislocki that his method promised to throw additional light on the development of the lymphatics. Whatever claims he has thus far made in this direction seem to be in accord, both for the development of the lymphatics and blood-vessels, with the old and disproved angioblast theory of His. In fact, he specifically states that the theory of the venous origin of the lymphatics which he claims to confirm, is in direct accord with the earlier work upon the development of blood-vessels by His.

My original plan has been greatly modified and extended, as so many other important questions presented themselves during the course of the investigation. In addition, therefore, to the question of lymphatic development, the above-mentioned inquiries, with one exception, will largely form the subject of this paper. This exception is a more detailed account of the development of the white blood-corpuscles, which may best be considered in a separate publication with illustrations.

2. METHODS

The following species of amphibia have been studied by the writer in connection with this investigation:

Larvae and adult of Rana pipiens, Schreber; larvae and adult of Rana sylvatica, Le Conte; year-old larvae and adult of Rana catesbiana, Shaw; adult of Rana clamata, Daudin, and Rana palustris, Le Conte; larvae of Acris (spec.?); advanced larvae of Diemyctylus viridescens, Rafinesque, and larvae and adult of Bufo lentiginosus, Shaw.

Trypan blue (Grübler) and dye No. 161 (Niagara blue)[1] in solutions of different concentrations as stated in the following pages, were the principal dyes used. The action of these two dyes on certain typical tissues was found to be identical.

The young frog and toad larvae were placed in solutions of trypan blue and dye No. 161 (Niagara blue), ranging in concentration from 1:800 to 1:2000. When treated in this manner a considerable number of them usually die at about the time the external gills begin to disappear. In dye solutions of low concentration such as 1:10,000 the larvae will live for an almost indefinite time. The action of such solutions, although slower than those of higher concentration, produces exactly the same results. The younger larvae were in all cases allowed to develop in solutions of dye which were made up fresh with ordinary tap water every twenty-four hours. On account of the scarcity of dye and number of experiments, it was often found necessary later in the Summer to make use of previously used solutions and, in the case of these older larvae, no difference in the action of the dye was observed.

In preparing larvae for section cutting which have remained in solutions of dye, it has been found desirable to transfer them from the dye solution to tap water, in which they should remain for at least twenty-four hours before killing and fixation. The object of this is to eliminate all of the dye from the alimentary canal which, if present after killing, often dissolves in the fixing agent and alcohols, and stains some of the tissues a deep and diffuse blue. In larvae so treated not a trace of dye will ordinarily be found in any of the tissues, except that which has actually been stored in the form of dye granules in the cytoplasm of the cell. In experimental larvae which have remained in tap water for several weeks before killing, dye granules may still be found stored in the cytoplasm of typical cells.

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[1] I am indebted to Dr. Paul A. Lewis of the Henry Phipps Institute for the dye No. 161, to whom my thanks are due.

Serial sections were made of three hundred and forty toad and frog larvae. These cover all of the critical stages before, as well as after definite reactions of the tissues toward the dye, in the form of dye granules, can be observed. Serial sections were also made of one hundred and ninety different tissues and organs of advanced larvae and adult amphibians which had been subjected to a treatment of dye.[2]

As fixing agents, a 10 per cent solution of neutral formalin and Bouin's fluid were almost exclusively used. A careful comparison of the tissues fixed by these two methods shows that dye granules remain unchanged in both cases and as originally deposited in the living cell. The only difference which results in the use of Bouin's fluid for the fixation of amphibian larvae lies, as far as I can determine, in the more perfect fixation of the cells.

Most of the larvae were stained *in toto* with borax-carmine. Mayer's alum-carmine and a 1 per cent aqueous solution of Bismarck brown were used for staining on the slide. A counter stain of eosin proved serviceable in some cases, and controls were often stained with Reagan's ('14) modification of Mann's methyl blue-eosin stain.

Observations were frequently made on larvae which had remained in dye solutions, to determine the rapidity and extent to which the lymphatics of the caudal fin had stored the dye. These observations were made in the usual manner with high power objectives on living larvae placed in a micro-aquarium. In Spalteholz preparations of the caudal fin in which the endothelium of the lymphatics has stored the dye, the dye granules could not be as distinctly observed as in the living animals.

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[2] All serial sections of larvae and of other tissues studied in connection with this investigation were prepared by my assistant Miss Grace Lloyd, to whom my thanks are due.

Experiments with trout and other fresh water teleost embryos show that when placed in colloidal acid dye solutions, the latter do not, as in amphibian larvae, gain entrance to the body and vitally stain the tissues. As observed by Wislocki ('16) for Hyla, the larvae of Acris are also less immune to the toxic action of these dyes than those of Rana and Bufo.

All of the microscopic observations referred to in the following pages were made with a Zeiss homogenous oil immersion objective (2 mm. apert. 1.40) and appropriate oculars.

3. ON THE DETERMINATION OF THE EARLIEST STAGE OF LARVAL DEVELOPMENT IN BUFO AND RANA, AT WHICH CERTAIN TYPICAL TISSUES ARE FIRST OBSERVED TO REACT TOWARD COLLOIDAL ACID DYES

Experiment 1 (table 1). Ova of Bufo lentiginosus were fertilized in the morning of May 31 and the first body movements observed in the evening of June 2. These larvae averaged four millimeters in length in the morning of June 3, at which time the external gills had not yet made their appearance. In the morning of June 3, the third day after fertilization, seventy-six (76) larvae were placed in a 1:1500 solution of Niagara blue (dye No. 161) made up with tap water, and were allowed to continue their development at ordinary room temperature. A certain number of larvae were removed daily from the dye solution from June 4 to June 14, inclusive, and, in each case, were transferred to tap water, in which they remained twenty-four hours before killing and fixation. Since the dye taken up by the circulation continues to act on certain tissues even after larvae have been transferred to tap water, the duration of its action is necessarily estimated in these experiments, from the time larvae are first placed in the dye until the time of killing and fixation. For example, the twelve larvae killed on June 9, referred to in column V of table 1, were placed in the dye solution on June 3, transferred from the latter to tap water on June 8 and killed on June 9. Experiment has shown that if these twelve larvae had been killed in the morning of June 8, instead of June 9, no indication of dye granules would likely have been observed in any cell of the body.

TABLE 1

Bufo lentiginosus

I EXP.	II DATE OF FERTILIZATION	III DATE OF IMMERSION IN DYE	IV NO EVIDENCE OF DYE GRANULES IN ANY TISSUE	V DYE GRANULES OBSERVED
1 (T^2)	May 31	June 3, 76 larvae, about 4 mm. in length Ext. gills not developed 1:1500 Niagara blue (dye No. 161)	June 5 (5 larvae, 6.5 mm. in length); Ext. gills present June 6 (5 larvae, 7 mm. in length); Ext. gills present June 7 (11 larvae, 8 mm. in length); Ext. gills lost June 8 (11 larvae, 8+ mm. in length);	
			June 9 (2 larvae, 8.5 mm. to 9 mm. in length)	June 9, 12 larvae
				June 10, 6 larvae
				June 11, 5 larvae
				June 12, 5 larvae
				June 13, 3 larvae
				June 14, 4 larvae
				June 15, 7 larvae

An examination of serial sections of five (5) of these larvae killed on June 5, five (5) killed on June 6, eleven (11) killed on June 7, eleven (11) killed on June 8 and two (2) killed on June 9, showed that no granules had been stored in the cytoplasm of any cell in these thirty-four (34) larvae.

Fourteen (14) larvae were killed on June 9 (ninth day after fertilization) and sectioned. In two (2) individuals, as stated above, no dye granules were observed in the cytoplasm of any cell. In the remaining twelve (12) larvae, however, dye granules were invariably met with in the cytoplasm of the cell in certain typical tissues (table 1, column V).

In all of the larvae concerned in this experiment which were killed after June 9, six (6) on June 10, five (5) on June 11, five

TABLE 2

Bufo lentiginosus

I EXP.	II DATE OF FERTILIZATION	III DATE OF IMMERSION IN DYE	IV NO EVIDENCE OF DYE GRANULES IN ANY TISSUE	V DYE GRANULES OBSERVED
2	May 31	June 4, 33 larvae, about 5 mm. in length	June 6, 8 larvae	
			June 7, 5 larvae	June 7, 1 larva
(T^3A)			June 8, 7 larvae	June 8, 2 larvae
				June 9, 10 larvae
		Ext. gill rudiments present 1:1000 trypan blue		

(5) on June 12, three (3) on June 13, four (4) on June 14 and seven (7) on June 15), dye granules were invariably found in the cytoplasm of the cell in certain typical tissues (table 1, column V).

Experiment 2 (table 2). Thirty-three (33) larvae of Bufo lentiginosus (controls of Experiment 1) were placed in a 1:1000 solution of trypan blue on June 4, one day later than in Experiment 1, and four days after fertilization. These larvae measured about five millimeters in length and the external gills were just beginning to make their appearance. Certain of these larvae were transferred daily from the dye solution, from June 5 to June 8, inclusive, to tap water in which they remained twenty-four hours before killing and fixation.

An examination of serial sections of eight (8) larvae killed on June 6, five (5) killed on June 7 and seven (7) killed on June 8, showed that no dye granules had been stored in the cytoplasm of any cell in these twenty (20) larvae. In one (1) larva killed on June 7 and two (2) larvae killed on June 8 dye was stored in the epithelium of a few pronephric tubules. These three larvae formed the only exceptions, however, in which dye had been deposited in any of the tissues among the larvae of this experiment which were killed previous to June 9. In all of the ten (10) larvae which were killed on June 9 (ninth day after fertilization) dye granules were invariably found in the cytoplasm of the cell in certain typical tissues.

Experiment 3 (table 3). This experiment differs from Experiment 2 only in the circumstance that the larvae of Bufo were

TABLE 3

Bufo lentiginosus

I EXP.	II DATE OF FERTILIZATION	III DATE OF IMMERSION IN DYE	IV NO EVIDENCE OF DYE GRANULES IN ANY TISSUE	V DYE GRANULES OBSERVED
3 (T³B)	May 31	June 4, 31 larvae, about 5 mm. in length Ext. gill rudiments present 1:1000 trypan blue Developed in dark room	June 8, 11 larvae	June 9, 7 larvae June 10, 9 larvae June 11, 2 larvae June 12, 2 larvae

allowed to develop in a dark room and were never exposed to the light. The concentration of the dye solution (1:1000 trypan blue), the date of immersion (June 4) and the age of the larvae at the time of immersion were the same in both cases.

Thirty-one (31) larvae were involved in this experiment. Certain of these larvae were transferred daily from the dye solution to tap water, from June 7 to June 11, inclusive, in which they remained twenty-four hours before killing and fixation.

An examination of serial sections of eleven (11) larvae killed on June 8 showed that no dye had been stored in the cytoplasm of the cell in any tissue of the body.

In seven (7) larvae killed on June 9 (ninth day after fertilization), nine (9) killed on June 10, two (2) killed on June 11 and two (2) killed on June 12, dye granules were invariably found in the cytoplasm of the cell in certain typical tissues. No apparent difference was observed in the behavior of larvae which were allowed to develop in a dark or light room, as far as reactions toward dye are concerned.

If we summarize the above experiments, we find among the 140 larvae of Bufo placed on the third and fourth days after fertilization in solutions of colloidal acid dyes, that dye was stored in the tissues of only three of the 66 larvae killed at successive intervals previous to the ninth day. Also, that in all except 2 of the 31 larvae killed on the ninth day after fertilization, and in all of the 43 killed at successive intervals subsequent to this time, dye granules were invariably present in the cytoplasm of the cell in certain typical tissues.

TABLE 4

Rana pipiens

I	II	III	IV	V
EXP.	DATE OF FERTILIZATION	DATE OF IMMERSION IN DYE	NO EVIDENCE OF DYE GRANULES IN ANY TISSUE	DYE GRANULES OBSERVED
4	April 7, probable date of fertilization	April 11, 46 larvae, 4.5 mm. in length	April 12, 4 larvae April 13, 6 larvae April 14, 6 larvae	
(L)		Ext. gills not developed in all cases	April 15, 8 larvae April 16, 6 larvae	
		1:2000 trypan blue (April 11 to April 16); changed to 1:1000 trypan blue on April 16	April 17, 6 larvae	April 17, 1 larva April 18, 4 larvae April 19, 2 larvae April 21, 3 larvae

Experiment 4 (table 4). Forty-six (46) larvae of Rana pipiens, measuring about 4.5 millimeters in length, were placed in a 1:2000 solution of trypan blue on April 11 and transferred to a 1:1000 solution on April 16. At the time they were first placed in the dye solution the external gills had not made their appearance in all of the larvae, so that the stage of development at time of immersion was approximately similar to that of the toad larvae in Experiment 1. As the frog larvae were brought into the laboratory at an early cleavage stage, the exact date of fertilization is unknown, but probably occurred in the morning of April 7. All of the larvae of this experiment were killed immediately on removal from the dye solution, with the result that the dye in the alimentary canal dissolved in the reagents and stained some of the tissues diffusely. For this reason the tissues are not as favorable for detailed study as those of larvae which have remained in tap water twenty-four hours before killing. I have been compelled to omit two other similar sets of experiments made on Rana on account of my neglect to rid the alimentary canal of dye before killing and fixing the larvae.

An examination of serial sections made of four (4) larvae of Rana killed on April 12, six (6) killed on April 13, six (6) killed on April 14, eight (8) killed on April 15, six (6) killed on April 16 and six (6) killed on April 17, showed that no dye had been stored in the cytoplasm of any cell in the body.

In one (1) larva killed on April 17, four (4) larvae killed on April 18, two (2) killed on April 19 and three (3) killed on April 21, dye granules were invariably found stored in the cytoplasm of the cell in certain typical tissues (table 4).

Although the number of individuals involved in this experiment is not especially large, like the experiments with toad larvae, it shows that no indication of dye granules becomes evident until the larvae have remained for at least six days in the dye. In view of certain other experiments about to be mentioned, this is a feature of considerable significance.

The writer has also studied the behavior of cleavage and early medullary groove stages of Bufo and Rana and finds that none of the cells store the dye at this time. *These experiments together with those above-mentioned clearly indicate that the initiation of the process or processes by which dye particles are stored in the cytoplasm of certain cells, must be primarily associated with the attainment of some distinctive ontogenetic larval stage, rather than with the length of time larvae are acted upon by dyes.*

Before proceeding to experiments which fully bear out this view, it is desirable to consider the reactions of the tissues toward colloidal acid dyes as observed in larvae of Bufo and Rana at this critical ontogenetic stage.

Under the conditions of the above-mentioned experiments, this critical ontogenetic stage in Bufo falls approximately on the ninth day after fertilization, while in Rana pipiens it probably falls on the tenth or eleventh day (table 4).

4. REACTIONS OF THE TISSUES IN BUFO AND RANA TOWARD COLLOIDAL ACID DYES, AS OBSERVED IN LARVAE WHEN KILLED AT THE CRITICAL ONTOGENETIC STAGE

An examination of serial sections of larvae of Bufo killed at this critical stage (ninth day after fertilization), shows that while the storage of dye granules by typical tissues has been quite extensive in some larvae, in others it has been exceedingly slight. Dye granules were found stored in the cytoplasm of the cell and never in the nucleus of the following organs and tissues of Bufo on the ninth day after fertilization:

1. *Pronephros.* Dye granules were found in the epithelium of the proximal portion of some of the tubules in the pronephros. In a few larvae the pronephric epithelium was the only tissue in the body in which the dye had been stored. The tubules which are the first to store the dye occupy a dorsal and lateral position in the pronephros. The epithelium of the pronephric (Wolffian) ducts and nephrostomal canals does not store the dye.

2. *Epithelium of pharynx.* Dye granules were found in thickened patches of epithelium which occupy a caudal and lateral position in the roof of the pharynx. They were also present in the epithelium covering a prominent fold which projects laterally, on each side, from the floor of the pharynx, near the caudal end of the tongue anlage. These epithelial areas of the pharynx, like the epithelium of the pronephric tubules, function as important regions for the deposition of dye particles. This function of the pharyngeal epithelium can be especially well demonstrated in much older larvae which have been subjected to a long immersion in dye.

Although the absorption of dye particles by such tissues as pharyngeal and pronephric epithelium may not be regarded in the same light as their ingestion by macrophages, it is an interesting fact that both processes are initiated in the embryo at the same time and in association with the attainment of the same ontogenetic larval stage.

3. *Stellate cells of the liver capillaries* (Kupffer's cells). Dye granules were found in the cytoplasm of the endothelial cells which line the venous capillaries of the liver (stellate cells of v. Kupffer). The parenchyma of the liver was never observed to house the dye nor even to be diffusely stained in larvae which had remained in tap water for some time before killing. In one instance Kupffer's cells were found to be the only cells in the body which had stored the dye in the form of granules. The possible significance of this condition will be considered in connection with another topic (Chapter 9).

4. *Endothelium of the lymphatic system.* Dye granules were stored in the endothelium of all the lymphatics, except in that which lines the anterior lymph-hearts. The endothelium of the

latter was never observed to be actively phagocytic. The posterior lymph-hearts are not developed on the ninth day after fertilization in Bufo.

5. *Endothelium of certain veins and aortic arches.* Dye granules were found in the cytoplasm of the endothelial cells of the renal-portal veins, which include Jacobson's vein (postcardinal) and the postcava (fused subcardinals). They were also found in the endothelium of the postcardinals anterior to the mesonephros and in the endothelium of the veins of the pronephros. The endothelium of the portal vein and its tributaries in the mesentery was found to be phagocytic; also that of a tributary of the portal vein which came from the pancreas. Dye granules were occasionally housed in an endothelial cell of the external and inferior jugular veins and occasionally in an endothelial cell of certain aortic arches. The endothelium of none of these three vessels can be regarded, however, as being consistently phagocytic. The aortic arches near the heart are frequently stained a diffuse deep blue in larvae in which the body is over-loaded with dye. This stain is usually removed in the living animal after the latter has remained in tap water for twenty-four hours before killing, and is not to be confused with the presence of dye granules in the endothelium of the aortic arches above-mentioned.

With the exception of the instances mentioned above, no dye granules were observed in the endothelium of any other veins or arteries in the body. The endothelium of the peripheral arteries and veins and of the dorsal aorta and its branches did not present the slightest indication of possessing a phagocytic function.

The tendency shown by the endothelium of certain lymphatics, veins and even arteries to ingest and store the dye, certainly suggests the possibility that vascular endothelium in general may have been primitively phagocytic. It is especially interesting to note in this connection that trypan blue is stored by the endothelium of the renal-portal veins in the adult carp (Wislocki, '17); also by the endothelium of the larger blood-vessels within the gills and by the renal and digitiform gland endothelium of an adult selachian (Hoskins, '17).

It is quite probable that the phagocytic character of the lymphatic endothelium observed in certain fishes (Wislocki, '17) and amphibia, may be correlated with the absence of lymph glands in these animals. A comparative study of the conditions observed in the embryo and adult of vertebrates in general might lead to interesting conclusions.

6. *Free cells.* Free cells containing dye granules were rarely found in Bufo on the ninth day after fertilization. A few mononuclears[3] containing dye granules were found in the mesenchyme, mesentery, intertubular tissue of the pronephros and in one instance in the portal vein. A few cells much larger than the mononuclears and usually of irregular form, were being desquamated from the endothelium of the cephalic lymph sinuses in some of the larvae killed on the ninth day after fertilization. These cells were loaded with dye granules and were found in the cephalic lymph sinuses and in the neighboring mesenchyme.

No trace of dye, in the form of granules, was observed in the tissues of twenty-nine larvae of Bufo killed on the ninth day after fertilization, other than those mentioned above.

The left mesonephros is not developed to any extent in Bufo lentiginosus on the ninth day after fertilization, and not a trace of dye was housed in the epithelium of the right mesonephros at this time. None of the red blood-cells, the thymus or thyroid glands, nor any of the tissues of the nervous system contained a trace of dye. No indication of the anlage of the spleen can be observed at this time.

Observations on the larvae of Rana pipiens (table 4) killed at the critical stage, are unfortunately not as extensive or as detailed as those described above for Bufo. If a larger number of larvae had been examined, probably the same average results would have been obtained for Rana as for Bufo, with the exception that in Rana, according to Wislocki, no endothelium, except that of the lymphatic system, apparently stores the dye. This is a question, however, which should be further investigated.

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[3] The term mononuclear leucocyte is applied here and in all subsequent cases mentioned in this paper, to cells essentially similar to those figured by Kliene-berger and Carl as "Mononukleärer L" and "Eingeschnürter mononukleärer L" in their treatise entitled "Die Blut-Morphologie der Laboratoriums-Tiere," Leipzig, 1912. (Tafel XII, 7 and 8 in fig. 1.)

Mononuclear leucocytes with eccentrically situated oval nuclei appear to be the chief free macrophages found in the tissues of anura which most readily react toward colloidal acid dyes. In later larval stages and in the adult they are found in abundance in the tissues of the frog and toad where they take up colloidal acid dye particles with great avidity.

In one of the seven (7) larvae of R. pipiens killed on April 17 (6 days in dye) and in four (4) killed on April 18, only the epithelium of a few tubules in the pronephros and the stellate cells of the liver capillaries had stored the dye. No trace of dye was observed in any other tissue of the body. In all of the larvae killed on April 19 and 21, dye granules were found in the endothelium of the lymphatics, in addition to the tissues mentioned above. No dye was stored, however, in the epithelium of the tubules in the mesonephros. See table 4.

5. EXPERIMENTS TO DETERMINE THE RATE AT WHICH THE STORAGE OF DYE GRANULES MAY BE ACCOMPLISHED IN LARVAE OF BUFO WHICH HAVE BEEN PLACED IN A SOLUTION OF COLLOIDAL ACID DYE AT THE CRITICAL ONTOGENETIC STAGE

When larvae of Bufo were placed in a solution of colloidal acid dye at intervals soon after fertilization, we have seen that dye granules do not ordinarily become visible in the cytoplasm of the cell, until the ninth day. If the storage of dye granules is associated with the attainment of a distinctive ontogenetic larval stage, and is not influenced by the length of time larvae remain in dye, we might expect the storage of dye granules to be considerably hastened when larvae are placed in dye solutions at the critical ontogenetic stage.

Experiment 5 (table 5). Forty (40) larvae of Bufo (controls of Experiment 1) were placed in a 1:1500 solution of trypan blue on the ninth day after fertilization (June 9).

In one (1) larva of this experiment killed on June 9, after five (5) hours in dye and five (5) hours in tap water, and in four (4) larvae killed on June 9, after five (5) hours in dye and twelve (12) hours in tap water, no dye granules were observed in the cytoplasm of any cell in the body.

TABLE 5

Bufo lentiginosus

I EXP.	II DATE OF FERTILIZATION	III DATE OF IMMERSION IN DYE	IV NO EVIDENCE OF DYE GRANULES IN ANY TISSUE	V DYE GRANULES OBSERVED
5	May 31	June 9, 40 larvae, about 8.5 mm. in length	June 9 (5 hours in dye, 5 hours in tap), 1 larva	
(T^4)		1:1500 trypan blue	June 9 (5 hours in dye, 12 hours in tap), 4 larvae	
			June 11, 6 larvae	June 11, 2 larvae
			June 12, 1 larva	June 12, 5 larvae
				June 13, 4 larvae
				June 14, 4 larvae
				June 15, 2 larvae
				June 16, 2 larvae
				June 17, 2 larvae
				June 19, 1 larva
				June 20, 3 larvae
				June 21, 2 larvae
				June 23, 1 larva

Among eight (8) larvae killed on June 11, after one (1) day in dye and one (1) day in tap water, dye granules were found to be present in the epithelium of the pronephric tubules and in the stellate cells of the liver of two (2) larvae. In the remaining six (6) larvae no trace of dye was observed in the cytoplasm of any cell in the body.

Among six (6) larvae killed on June 12, after two days in dye and one day in tap water, dye granules were found in the typical tissues of five (5) individuals. In the remaining larva no dye granules were observed.

In the remaining twenty-one (21) larvae which were killed on successive days, between June 13 and June 23, dye granules were invariably found in the cytoplasm of certain typical cells.

This experiment shows that considerable variation exists as regards the rate at which the different individuals of this group of larvae had developed prior to their immersion in dye on June 9. It also shows in the case of two of the more advanced larvae that the storage of dye granules was accomplished within two days, and that in all but one of the six individuals killed on June 12 (column IV, table 5) it was accomplished within three days.

In larvae killed subsequent to June 12, dye granules were invariably found.

We know by experiment that the storage of dye granules by certain typical tissues is generally potentially possible on the ninth day after fertilization in Bufo. The circumstance that the process has been prolonged in some of these larvae, appears to be due not only to a difference in their degree of development at the time of immersion, but also to the circumstance that conditions at this time are less favorable for the rapid ingestion or absorption of colloidal dye particles than at a more advanced age.

If this view is correct it should be borne out by experiments on more advanced larvae in which a storage of dye granules should be accomplished within a still shorter time.

6. EXPERIMENTS WHICH SHOW THAT THE STORAGE OF DYE GRANULES MAY BE ACCOMPLISHED IN A RELATIVELY SHORT TIME, WHEN LARVAE ARE PLACED IN SOLUTIONS OF COLLOIDAL ACID DYES AT AN ADVANCED STAGE OF DEVELOPMENT

All of the larvae used in these experiments are so advanced in development, that no doubt can exist that their tissues and organ systems have attained a high state of differentiation.

Experiment 6. (*a*) A number of toad larvae were placed in a 1:1500 solution of Niagara blue on August 16 (77 days after fertilization). After twelve hours and fifteen minutes immersion in the dye, some of the lymphatics of the caudal fin appeared to be stained a light blue in one of the larvae. An examination of the living larva showed that dye granules were beginning to be stored in the cytoplasm of the lymphatic endothelial cells. The liver and mesonephros were immediately removed from the body and sectioned. No dye granules were observed in the cytoplasm of the stellate cells of the liver, or in the epithelium of the mesonephric tubules. A few mononuclears containing dye granules were found, however, in the capillaries of the liver and in the intertubular tissue of the mesonephros. The mononuclears in the kidney were more deeply stained than those found in the liver capillaries.

b) In another individual of this same experiment which had remained in the dye solution for twenty hours and fifteen minutes, a few of the lymphatics of the caudal fin appeared to be more deeply stained by the dye, than in the larva above-mentioned. The liver and mesonephros were removed from the body and the liver sectioned. No definite traces of dye granules were observed in the stellate cells of the liver, but a few mononuclear leucocytes containing dye granules were found in its capillary vessels. A teased preparation of the kidney showed that the dye had been stored in the epithelium of a few tubules, and mononuclear cells containing dye granules were also present in the intertubular tissue.

These two individuals by no means represent the average conditions observed in the entire lot of larvae concerned in this experiment. They were chosen from the lot solely on account of the presence of dye which was observed in the lymphatics of the caudal fin. In none of the other larvae of this experiment were the lymphatics of the caudal fin stained by the dye when the above observations were made. After a short immersion in dye, larvae which may appear slightly bluish in color are more liable than others to have dye granules stored in their tissues.

Experiment 7. Similar observations were made on unidentified species of advanced frog larvae which were brought into the laboratory during July and August.

a) Frog larvae averaging about 20–22 millimeters in length, were placed in a 1:1000 solution of trypan blue on August 10. After sixteen and one-half hours immersion in the dye solution, the liver and mesonephros were removed from one of the larvae in which a doubtful trace of dye was observed in one of the lymphatics of the caudal fin. A study of serial sections of the liver and mesonephros showed that no dye had been stored in the stellate cells of the liver, or in the epithelium of the tubules in the mesonephros. A few mononuclears containing dye granules were found, however, in the liver capillaries and in the intertubular tissue of the kidney. The mononuclears found in the liver were more deeply stained than those in the kidney.

b) After remaining fifteen hours and forty minutes in the dye solution, the liver and mesonephros were removed from the body of two other individuals chosen at random, in which no indication of dye granules could be observed in the lymphatics of the caudal fin. A study of sections showed that a trace of dye had been stored in the stellate cells of the liver in one individual, and that no dye had been stored in the liver of the other. It was also found that no dye had been stored in the epithelium of the mesonephric tubules in either larvae. In both larvae, however, mononuclears containing dye granules were found in the liver capillaries and in the intertubular tissue of the kidneys.

Experiments 6 and 7 show when an advanced stage of larval development has been reached, that the storage of dye granules in the cytoplasm of typical cells no longer becomes a question of days, but may be accomplished in a relatively few hours. This bears out the view previously expressed, that the prolongation of the ingestion process at the critical ontogenetic stage, at which dye granules are ordinarily first observed, is probably due to the circumstance that developmental conditions are less favorable for rapid ingestion at this time, than at a more advanged age (Exp. 5).

These experiments would therefore seem to confirm the view that the initiation of the process by which dye granules are stored in the cytoplasm of certain cells is associated with the attainment of a distinctive ontogenetic larval stage. Experiment has shown that tissues do not react toward these dyes until this stage is reached, irrespective of the length of time larvae may remain in dye before this time.

These experiments also show that the mononuclear leucocytes may store the dye prior to all other typical tissues, and that the endothelium of the lymphatics of the caudal fin may even store the dye before the epithelium of the mesonephric tubules or the stellate cells of the liver capillaries. It is needless to state and, as experiment shows, if these same larvae had remained a slightly longer time in the dye, the storage of dye granules by the typical tissues would have been still more extensive. See Experiments 8 b and 11.

It is difficult matter to lay down a rule which would answer in all cases, regarding the shortest time required for dye to be stored in all of the typical tissues. It may be stated, however, in such advanced larvae as those mentioned above, that dye granules will usually be found in many of the typical tissues, after twenty-four hours immersion in dye (1:1000) and twenty-four hours in tap water before killing. As a guide to those who wish to repeat the experiment, forty-eight hours is the lowest average limit I would care to assign in the circumstances, as considerable individual variation is met with. This is well shown on table 6 which illustrates the variable length of time required in different individuals for the lymphatics of the caudal fin to store the dye. The extent to which dye has been stored in the caudal fin lymphatics may be regarded, in general, as an index of the relative extent of its storage in other typical tissues. Cases in which no dye has been stored in the caudal fin lymphatics do not necessarily signify, however, that it may not have been stored elsewhere in the body. See Experiment 7 b.

In larvae of R. temporaria which had been placed in solutions of trypan blue (1:800–1:1600) at time of hatching, Wislocki ('16) observed on about the fourth day of immersion faintly discernible traces of dye to be present in the lymphatics of the caudal fin. On the eighth day of immersion he found all of the lymphatics of the fin to be deeply stained.

In may be of interest to compare the above observations of Wislocki with those of the writer found on table 6.

All of the above-mentioned experiments have thus far been made upon relatively young larvae. The two following experiments (Experiments 8 and 9) show that the method of vitally staining typical tissues by immersing young larvae in solutions of acid dyes, can also be applied to year-old larvae of R. catesbiana and to adult anura.

TABLE 6

Showing rate at which endothelium of the caudal fin lymphatics may store the dye in advanced toad and frog larvae (July and August)

EXP.	LARVAE	DYE SOLUTION	LENGTH OF IMMERSION IN DYE	LYMPHATICS OF CAUDAL FIN AS OBSERVED IN INDIVIDUAL CASES
T^{11}	Toad	1:1500 dye 161	12 h. 15 min.	Few lightly stained
T^{11}	Toad	1:1500 dye 161	20 h. 15 min.	Few brilliantly stained
T^{10}	Toad	1:800 trypan blue	22 h. 40 min.	Trace of dye in few
T^{10}	Toad	1:800 trypan blue	30 h.	Few brilliantly stained
T^{10}	Toad	1:800 trypan blue	46 h.	All brilliantly stained
R^{3}	Frog	1:1000 trypan blue	16½ h.	Doubtful trace of dye in few
R^{4}	Frog	1:1000 trypan blue	23½ h.	Few slightly stained
R^{4}	Frog	1:1000 trypan blue	24 h.	Doubtful trace of dye in one
R^{4}	Frog	1:1000 trypan blue	30 h.	Trace of dye in few
R^{1}	Frog	1:1000 trypan blue	36 h.	Lightly stained
R^{1}	Frog	1:1000 trypan blue	36 h.	All brilliantly stained
R^{3}	Frog	1:1000 trypan blue	40 h.	All brilliantly stained
R^{5}	Frog	1:1500 dye 161	40 h.	Few brilliantly stained
R^{1}	Frog	1:1000 trypan blue	40 h.	Lightly stained
R^{6}	Frog	1:1000 trypan blue	36 h.	Some brilliantly, some lightly and others unstained
R^{2}	Frog	1:1000 trypan blue	52 h.	Lightly stained
R^{1}	Frog	1:1000 trypan blue	69 h.	All brilliantly stained
R^{2}	Frog	1:1000 trypan blue	76 h.	Some brilliantly stained; others not

7. EXPERIMENTS WHICH SHOW THAT THE METHOD OF VITALLY STAINING TYPICAL TISSUES BY IMMERSING YOUNG LARVAE IN SOLUTIONS OF COLLOIDAL ACID DYES, CAN ALSO BE APPLIED TO YEAR-OLD LARVAE OF R. CATESBIANA AND TO ADULT ANURA

A. Year-old larvae of R. catesbiana (Solutions of low concentration)

Experiment 8. Four year-old tadpoles of R. catesbiana were placed in a 1:1500 solution of trypan blue in the morning of June 26. The liver and mesonephros were removed daily from one of the tadpoles and sectioned.

a) The tadpole killed on June 27 (1 day in dye) measured sixty-five millimeters in length. Serial sections showed that no dye had been stored in the stellate cells of the liver or in the epithelium of the mesonephric tubules. A large lightly stained mononuclear cell containing fine dye granules was occasionally found in the intertubular tissue of the kidney.

b) On June 28 (2 days in dye) dye granules were observed in the epithelium of a few of the tubules in the mesonephros and a mere trace of dye was found in some of the stellate cells of the liver capillaries. Mononuclears containing dye granules were occasionally found in the capillaries of the liver and in the intertubular tissue of the kidney. This larva measured eighty-two millimeters in length.

c) On June 29 (3 days in dye) the stellate cells of the liver capillaries were more deeply stained than in the tadpole killed on June 28, and dye granules were also abundant in the epithelium of certain tubules in the mesonephros. Mononuclear cells containing dye granules were found in the liver capillaries and in the intertubular tissue of the mesonephros. This tadpole possessed rudimentary hind limbs and measured eighty-two millimeters in length.

d) On June 30 (4 days in dye) dye granules were abundantly distributed in the cytoplasm of the stellate cells of the liver capillaries and in the epithelium of certain tubules in the mesonephros. Deeply stained mononuclear leucocytes were also present in the liver capillaries, intertubular tissue of the kidneys and in the spleen. This tadpole possessed rudimentary hind limbs and measured ninety-four millimeters in length.

It will be observed that these experiments still further bear out the view that the storage of dye granules may be accomplished in a relatively short time, when larvae are placed in acid dye solutions at an advanced stage of development.

B. Adult anura (*Solutions of high and low concentration*)

Experiment 9 a). An adult toad (Bufo lentiginosus) was allowed to remain in a finger bowl containing a 1:100 solution of trypan blue for several hours daily between July 16 and July 22, inclusive, and killed on July 23. The actual length of time the toad remained in dye during the course of seven days was sixty-one hours.

On removing the skin from the abdomen the muscles presented a bluish-green appearance. The alimentary canal was filled with dye and its walls were stained intensely blue. All of the joints and tendinous sheaths near the latter were stained a deep blue. The roof of the mouth and walls of the pharynx were also diffusely stained. The veins and lymphatics of the mesentery were deeply stained but no dye granules were observed in the cytoplasm of their endothelium. The aortic arches near the heart were also diffusely stained. The lungs, testes, fat bodies, nerves and central nervous system were unstained. No white blood-corpuscles containing dye granules were observed in the circulation. Serial sections were made of the liver, kidneys and spleen.

A study of sections showed that dye had been stored in abundance in the cytoplasm of the stellate cells of the liver capillaries and in the epithelium of certain tubules in the kidneys, but no trace of dye was observed in the spleen. Mononuclear cells containing dye granules were found in the intertubular tissue of the kidneys.

b) An adult frog (R. pipiens) was placed in a finger bowl containing a 1:1500 solution of trypan blue on April 10, in which it remained continuously until April 26, when killed. The liver, kidneys and testes were removed from the body and sectioned.

Dye granules were stored in abundance in the stellate cells of the liver capillaries and in the epithelium of certain tubules in the kidney. They were also observed in the interstitial tissue of the testis. Numerous mononuclear cells containing dye granules were found in the intertubular tissue of the kidney.

c) An adult frog (R. clamata) was allowed to remain in a finger bowl containing a 1:200 solution of Niagara blue (dye No. 161) for several hours daily between September 12 and September 28, inclusive, and killed on October 5. The actual length of time the frog remained in the dye during the course of twenty-three days was seventy-four hours.

The general appearance of the muscles, organs, etc., was essentially the same as that mentioned in Experiment 9 a. A study of sections showed that dye had been stored in abundance in the cytoplasm of the stellate cells of the liver and in the epithelium of certain of the tubules in the kidneys. Mononuclear cells containing dye granules were also found in the intertubular tissue of the kidneys.

8. EXPERIMENTS TO DETERMINE THE PORTAL OF ENTRY OF THE DYE

In all of the preceding experiments the method employed of immersing the larvae and adults in solutions of dye, does not permit us to determine definitely how the dye gains admission to the body. As dye is taken into the alimentary canal through the mouth in such cases, there is an equal opportunity for it to pass through the intestinal mucosa as through the integument.

Wislocki ('16 a, p. 128) believes it gains admission to the body in the larvae of Rana through the alimentary canal and speaks as follows on this question:

> Study of the sections seems to show conclusively that the epidermis does not serve as a portal of entry for the dye, since there are no visible traces of trypan-blue within or between the cells of this tissue. It seems more probable that the stain gains admission to the body through the alimentary canal, because in certain regions of the gut, the entire intestinal mucosa and submucosa are colored a deep diffuse blue and occasionally definite granules of dye are distinguishable in these layers. The embryonic intestine behaves radically differently towards the dye than does that of the adult, which under no circumstance, has been known to absorb the dye or to have visible particles of stain in the cells of the mucosa.

The experiments of Overton ('04) and Maxwell ('13) point to the conclusion that there is a constant flow of water through the skin in the case of a frog immersed in water and that the excess of water is eliminated by the kidneys. It has been stated, according to Bayliss ('15, p. 356), that the urine secreted by a frog in twenty-four hours may exceed the total weight of the body. After the water has passed through the skin Volhard ('12) has shown that it is conveyed to the general circulation by the lymphatics. The experiments of Moore ('15) also point to the fact that the water is taken up by the veins as well as by the lymphatics. Be this as it may, it is an assured fact that water passes through the frog's skin in accordance with the laws of osmosis, and that it reaches the general circulation from the intercellular tissue spaces by way of the lymphatics.

By means of two sets of experiments, the writer has been able to determine definitely the portal of entry of the dye in both larval and adult anura; by the simple method of immersing only a portion of the body of the larva or adult in the acid dye solution, and by feeding experiments on adults.

A. Suspension of only a portion of the body in a solution of colloidal acid dye

It was found that neither larvae nor adults endure a prolonged and continuous treatment of this character. It was therefore found necessary to subject them at intervals to a number of short immersions, after which they were immediately transferred to aquaria. The amount of dye which enters the body in such cases is naturally limited, and this method does not indicate the extent to which the tissues might have stored the dye within the time limits of the experiment if the suspension in dye had been continuous.

Every precaution was taken to prevent the possibility of any dye reaching the alimentary canal through the oral cavity. The tails of the smaller larvae were suspended in the dye through the meshes of a mosquito netting, while brass funnels were used for the larger larvae of R. catesbiana. Adult frogs and toads were bound to glass rods with wide-meshed bandages. This makes it possible to suspend the legs and body in the dye to the extent desired. The pressure of the bandage on the body undoubtedly obstructs the lymph flow to some extent, but this is unavoidable. In the case of all adult frogs and toads, from twelve to twenty-four hours were allowed to elapse between the time of the last suspension in dye and the time of killing.

Experiment 10. (Partial immersion of young larvae in solutions of low concentration).

a) The tail of a larva of R. sylvatica was suspended for fifteen minutes on May 11 in a 1:1000 solution of trypan blue and the larva then transferred to tap water for twenty-four hours before killing. A study of serial sections showed that no dye had been stored in any tissue of the body.

b) The tail of a larva of R. sylvatica was suspended for about fifteen minutes on May 11 and 12 in a 1:1000 solution of trypan blue and the larva killed in the morning of May 13. A study of serial sections showed that no dye had been stored either in the cytoplasm of the stellate cells of the liver or in the epithelium of the tubules in the pro- and mesonephros. Certain of the tubules in both kidneys, however, presented a bluish appearance which indicated that dye had reached the circulation.

c) The tail of a larva of R. sylvatica was suspended for about twenty minutes daily on three consecutive days (May 11, 12 and 13) in a 1:1000 solution of trypan blue and the larva killed in the morning of May 14. Serial sections showed the unmistakable presence of dye granules in the cytoplasm of the stellate cells of the liver and in the epithelium of a number of tubules in the pro- and mesonephros. The above-mentioned larvae were about thirty-three days old on May 11 and may be regarded as fairly advanced larvae.

Experiment 11. (Partial immersion of larvae in solutions of high concentration).

The caudal fourth of the tails of two larvae of R. pipiens (34 days after fertilization) were suspended in a 1:100 solution of trypan blue for one-half hour in the morning and afternoon of May 3, and the larvae then transferred to tap water. Both larvae were killed in the morning of May 4, after remaining about twenty-four hours in tap water. The portion of the tail which had been submerged in dye sloughed off during the night, but at time of killing the wound had healed and both tadpoles were very active. Before the larvae had been suspended in the dye the heart beat was about one hundred and fourteen to the minute. After one-half hour suspension in dye the heart beat was reduced to ninety.

An examination of serial sections of both larvae showed that dye granules had been deposited in abundance in the stellate cells of the liver and in the epithelium of the pro- and mesonephros. Dye granules were also found in the endothelium of some of the lymphatics, but were not observed in that of the large ventral cephalic lymph sinus.

This experiment, when compared with Experiments 6 and 7, is especially interesting by showing the rapidity with which typical tissues may react toward acid dyes, when advanced larvae are subjected to solutions of high concentration. The amount of dye stored in the tissues of these two larvae is relatively large, so that the storage of dye particles must have begun soon after their suspension in the dye solution.

Experiment 12. Compare with Experiments 6, 7, 8 and 9. (Partial immersion of year-old larvae of R. catesbiana in solutions of colloidal acid dyes).

a) The tail of a year-old tadpole of R. catesbiana was suspended for one-half hour in a 1:100 solution of trypan blue in the morning and afternoon of July 23, and the tadpole killed in the morning of July 24. Sections were made of the liver and mesonephros. Not a trace of dye was observed in the liver. Certain tubules of the mesonephros were stained a light blue, in the epithelium of which a few dye granules were occasionally met with. A few mononuclear cells containing dye granules were also observed in the intertubular tissue of the mesonephros.

b) The tail of a year-old tadpole of R. catesbiana was suspended twice daily for one-half hour on July 23 and 24, in a 1:100 solution of trypan blue and the tadpole killed in the morning of July 25. Sections were made of the liver and mesonephros. Dye granules were found in a number of the stellate cells of the liver, and large mononuclears containing dye granules were also observed in the liver capillaries. A number of tubules in the mesonephros were deeply stained and dye granules were abundant in their epithelium. Numerous deeply stained large mononuclear cells were also found in the intertubular tissue of the mesonephros.

c) The tail of a year-old tadpole of R. catesbiana was suspended twice daily for one-half hour on July 20, 21 and 22 in a 1:100 solution of trypan blue and the tadpole killed in the morning of July 23. Sections were made of the liver, mesonephros and spleen. Dye granules were found in a number of the stellate cells of the liver and in the epithelium of several of the tubules in the mesonephros. Deeply stained mononuclear cells were abundant in the intertubular tissue of the mesonephros and a few more lightly stained ones were found in the spleen.

TABLE 7

Partial immersion of body of adult frogs in solutions of colloidal acid dyes

EXP.	SPECIES	DURATION OF EXPERIMENT	LENGTH OF TIME SUSPENDED IN DYE	
13a (G^{3})	R. palustris	3 days, May 30 to June 2	1 h. 45 min. in 1:100 trypan blue	No dye granules observed
13b (G^{5})	R. palustris	4 days, July 5 to July 9	3 h. 45 min. in 1:100 dye No. 161	Dye granules observed in typical tissues
13c (G^{11})	R. catesbiana	5 days, July 19 to July 24	14 h. 45 min. in 1:100 trypan blue	
13d (G^{13})	R. palustris	6 days, July 19 to July 25	21 h. 25 min. in 1:150 trypan blue	
13e (G^{7})	R. clamata	10 days, July 9 to July 19	11 h. 15 min. in 1:100 dye No. 161	

Numerous other experiments of this character were made on year-old tadpoles of R. catesbiana which leave no room for doubt that solutions of colloidal acid dyes can gain entrance to the body through the integument.

In other experiments the treatment of short immersions of the tail was extended over a period of seven, eight, nineteen, twenty-two and even seventy-five days. In the latter case the tail was suspended twice daily in a 1:100 solution of trypan blue, only between July 20 and July 26, and the tadpole killed on October 4. Although over two months had elapsed between the last treatment and the date of killing, dye was found in abundance stored in typical cells of the body.

Experiment 13 (table 7). (Partial immersion of adult frogs in solutions of colloidal acid dyes).

In all of the following experiments, except Experiment 13 e, the hind legs and caudal half of the body were suspended in the dye solution.

a) An adult of R. palustris was suspended for short intervals daily from May 30 to June 1, inclusive, in a 1:100 solution of trypan blue and killed on June 2. The actual length of time the frog was suspended in the dye during a period of three days was one hour and forty-five minutes. No dye was stored in the liver and kidneys or in any other tissues as far as examined.

b) An adult of R. palustris was suspended for short intervals daily from July 5 to July 8, inclusive, in a 1:100 solution of Niagara blue (dye No. 161), and killed on July 9. The actual length of time the frog was suspended in dye during a period of four days was three hours and forty-five minutes.

On removal of skin the muscles of the abdomen presented a greenish-blue appearance, while the muscles and joints of the hind limbs were stained a deep blue. The walls of the oral cavity and pharynx were deeply stained. The walls of the stomach and rectum were markedly bluish, but the small intestines remained unstained.

Sections showed the unmistakable presence of dye granules in the stellate cells of the liver and in the epithelium of a few tubules in the kidney. Not a trace of dye was observed in sections of the testis or spleen. Sections of the stomach showed the submucosa to be lightly, though diffusely stained. Mononuclear cells loaded with dye granules were observed in the mucosa of the stomach.

c) A young adult of R. catesbiana was suspended for short intervals daily from July 19 to July 23, inclusive, in a 1:100 solution of trypan blue and killed on July 24. The actual length of time the frog was suspended in dye during a period of five days was fourteen hours and forty-five minutes.

Serial sections were made of the liver, kidneys and spleen. Dye granules were stored in the stellate cells of the liver capillaries and in the epithelium of a few tubules in the kidney. Large deeply stained mononuclear leucocytes containing dye granules were found in the intertubular tissue of the kidneys and more lightly stained ones in the spleen.

d) An adult of R. palustris was suspended for short intervals daily from July 19 to 24, inclusive, in a 1:150 solution of trypan blue and killed on July 25. The actual length of time the frog was suspended in dye during a period of six days was twenty-one hours and twenty-five minutes.

The muscles of the legs and abdomen were decidedly bluish in appearance, also the joints of both fore and hind limbs. The walls of the stomach, distal half of the small intestine and rectum were lightly stained. The proximal half of the small intestine was unstained. The aortic arches near the heart and ventral surface of the vertebral column were deeply stained.

A study of sections showed that dye had been stored in abundance in the stellate cells of the liver capillaries and in the epithelium of a few of the tubules in the kidney. A precipitate of the dye was found in some of the venules of the spleen. The endothelium of these venules was deeply stained but it was impossible to determine the presence of dye granules on account of the density of the stain. No trace of dye was observed in sections of the testes or fat bodies. Mononuclear leucocytes containing dye granules were abundant. They were observed in sections of a number of tissues, including the mucosa of the duodenum. The presence of phagocytic cells among the epithelial cells of the frog's intestinal mucosa has already been observed by R. Heidenhain ('88), Bizzorero ('92) and others.

e) This experiment differs from those above-mentioned, in the circumstance that only the hind feet and ankles were suspended in the dye.

A small adult of R. clamata was suspended for short intervals daily from July 9 to July 18, inclusive, in a 1:100 solution of Niagara blue, and killed on July 19. The actual length of time the frog was suspended in dye during a period of ten days was eleven hours and fifteen minutes.

On the removal of skin the muscles and joints, except that of the ankle, were found to possess their natural color and with no indication of being stained. None of the viscera appeared to be stained in the slightest degree.

A study of sections showed that not a trace of dye had been stored in the stellate cells of the liver capillaries. The merest trace of dye, however, was unmistakably stored in the form of granules in the epithelium of a few tubules in the kidneys. Deeply stained mononuclear leucocytes containing dye granules were also found in the intertubular tissue of the kidneys.

The amount of dye which entered the circulation through the lymphatics of the feet and ankles must have been exceedingly

TABLE 8

Partial immersion of body of adult toads in solutions of colloidal acid dyes

EXP.	SPECIES	DURATION OF EXPERIMENT	LENGTH OF TIME SUSPENDED IN DYE	
14a (G^{6})	B. lentiginosus	3 days, July 7 to July 10	3 h. 37 min. in 1:100 dye No. 161	Dye granules observed in typical tissues
14b (G^{16})	Same	3 days, July 23 to July 26	3 h. 40 min. in 1:100 trypan blue	
14c (G^{16})	Same	4 days, July 23 to July 27	5 h. 30 min. in 1:100 trypan blue	
14d (G^{10})	Same	9 days, July 16 to July 25	25 h. 37 min. in 1:200 dye No. 161	

slight. It is important to note, however, as observed in a number of other experiments, that the mononuclear cells and the epithelium of the kidney appear to store the dye prior to the stellate cells of the liver, in all cases where the portal of entry of the dye is restricted to the integument.

Experiment 14 (table 8). (Partial immersion of body of adult toads in solutions of colloidal acid dyes).

In the following experiments only the hind legs and posterior half of the body were suspended in the dye.

a) A young adult of B. lentiginosus was suspended for short intervals daily between July 7 and July 9, inclusive, in a 1:100 solution of Niagara blue (dye No. 161) and killed on July 10. The actual length of time the toad was suspended in dye during a period of three days was three hours and thirty-seven minutes.

The muscles, joints and viscera did not show the slightest sign of being diffusely stained by the dye. Examination of sections showed that a doubtful trace of dye had been stored in a very few of the stellate cells of the liver capillaries, and that the epithelium of a few tubules in the kidney had stored the dye. One would likely have overlooked the presence of dye granules in both the liver and kidney, if serial sections of the entire organs had not been made. The most interesting feature of this experiment is that deeply stained mononuclear leucocytes containing dye granules were occasionally found in the intertubular tissue of the kidney, which shows that these cells must have been actively phagocytic prior to the Kupffer cells. No dye was stored in any cell of the spleen.

b) A young adult of B. lentiginosus was suspended for short intervals daily between July 23 and July 25, inclusive, in a 1:100 solution of trypan blue and killed on July 26. The actual length of time the toad was suspended in the dye during a period of three days was three hours and forty minutes.

On removing the skin some of the joints appeared slightly bluish but the muscles were unstained.

A mere trace of dye was stored in a few of the stellate cells of the liver capillaries and in the epithelium of a few of the tubules in the kidney. Deeply stained mononuclears containing dye granules were found in the intertubular tissue of the kidneys.

c) A young adult of B. lentiginosus was suspended for short intervals daily between July 23 and July 26, inclusive, in a 1:100 solution of trypan blue and killed on July 27. The actual length of time the toad was suspended in the dye during a period of four days was five hours and thirty minutes.

On removal of skin the knee joints appeared slightly bluish but the muscles and viscera were unstained.

A mere trace of dye was observed in a few of the stellate cells of the liver capillaries in the form of very minute and pale bluish granules. In teased preparations of the kidney some of the tubules were slightly bluish and sections showed that dye granules were stored in the epithelium of a few tubules. Deeply stained mononuclears containing dye granules were occasionally found in the intertubular tissue of the kidney.

d) An adult of B. lentiginosus was suspended for short intervals daily from July 16 to July 24, inclusive, in a 1:200 solution of Niagara blue (dye No. 161) and killed on July 25. The actual length of time the toad was suspended in dye during a period of nine days was twenty-five hours and thirty-seven minutes.

The muscles of the body and walls of the intestinal canal were quite deeply stained. The vessels in the mesentery, the joints and the ventral surface of the vertebral column were also stained a deep blue.

Dye was stored in the stellate cells of the liver capillaries, but not to the extent observed in Experiment 13 d. Dye was stored in abundance, however, in the epithelium of a number of tubules in the kidney and an occasional deeply stained mononuclear cell in the intertubular tissue was also found. Mononuclear cells containing dye granules were observed in the spleen. No dye was stored in the oviducts or ovary but an occasional mononuclear containing dye granules was found in the connective tissue of the lung.

The above experiments clearly show that solutions of colloidal acid dyes pass freely through the integument of both larval and adult frogs and toads, and that after reaching the general circulation the dye is stored in part by the kidneys, and in part by certain other typical tissues.

Experiments in which the portal of entry of the dye is restricted to the integument indicate that the mononuclear leucocytes are among the first, if not the first of the macrophages to store the dye, and that the epithelium of certain tubules in the kidneys also stores the dye prior to the stellate cells of the liver capillaries.

The above experiments were not made with the intention of determining the rapidity with which certain typical cells might react toward the dye in adult frogs and toads. When compared with Experiments 1, 2, 3 and 4, however, it is seen that they still further bear out the view that the storage of dye granules by typical cells may be accomplished in a much shorter time after, than before the critical ontogenetic larval stage has been reached. (Chapters 3, 5 and 7.)

B. Feeding experiments on adult frogs and toads (table 9)

It is difficult to state definitely the amount of dye which actually reached the intestinal canal at each feeding. Some individuals are good feeders, so to speak, and retain the dye, while others often eject a good deal of it after being fed. The essential feature in all of these feeding experiments is to get sufficient dye

TABLE 9

Feeding experiments on adult frogs and toads

EXP.	SPECIES	DURATION OF EXPERIMENT	NUMBER OF FEEDINGS	
15 (Y^{6})	R. clamata	10 days, June 5 to June 15	16, 1:1000 trypan blue	No dye granules observed
16a (Y)	R. pipiens	1 day, May 2 to May 3	2, 1:100 trypan blue	
16b (Y^{10})	R. catesbiana	5 days, July 20 to July 25	9, 1:100 trypan blue	Dye granules observed in typical tissues
16c (Y^{1})	R. pipiens	6 days, May 3 to May 9	12, 1:100 trypan blue	
16d (Y^{7})	R. clamata	7 days, June 18 to June 25	12, 1:200 and 1:100 trypan blue	
17a (Y^{11})	B. lentiginosus	3 days, July 27 to July 30	4, 1:100 trypan blue	No dye granules observed
17b (Y^{11})	Same	6 days, July 27 to Aug. 2	13, 1:100 trypan blue	Dye granules observed in typical tissues
17c (Y^{11})	Same	9 days, July 27 to Aug. 5	21, 1:100 trypan blue	
17d (Y^{9})	Same	11 days, July 12 to July 23	22, 1:100 trypan blue	
17e (Y^{11})	Same	15 days, July 27 to Aug. 11	32, 1:100 trypan blue	
17f (Y^{11})	Same	21 days, July 27 to Aug. 17	47, 1:100 trypan blue	
17g (Y^{9})	Same	28 days, July 12 to Aug. 9	55, 1:100 trypan blue	

into the intestinal canal, so that the chances of its absorption by the mucosa will be favorable. The frogs and toads were fed by means of a small pipette holding about 1 cc. of dye. In the case of the larger animals this may be regarded as approximately the amount given at each feeding.

Experiment 15 (table 9). Three experiments were made by feeding adult frogs with solutions of trypan blue of low concentration. In one extreme case (Exp. 15) a frog was fed sixteen times in the course of ten days but no indication of vital staining was observed in any of the typical tissues.

In all of the remaining experiments solutions of high concentration were used.

Experiments 16 and 17 (table 9). In two experiments (16 a and 17 a) in which the duration of the experiment was limited to one and three days respectively, and the number of feedings few, no vital staining was observed in any of the tissues.

In all of the remaining experiments, however, some or all of the typical tissues of the body were found to store the dye. The essential feature noticed in connection with these feeding experiments was that the stellate cells of the liver were invariably found to store the dye prior to the epithelium of the kidney tubules. In Experiments 16 d and 17 b dye was stored in the stellate cells of the liver, but not a trace was observed in the epithelium of the tubules in the kidney. In Experiments 16 b, 16 c, 17 c, 17 d and 17e a relatively large amount of dye was typically stored in the liver, while a mere trace only was found in the epithelium of the kidneys. Irrespective of the amount present, in every one of the above-mentioned cases the amount of dye stored in the stellate cells of the liver was invariably in excess of that found in the epithelium of the tubules in the kidney.

In Experiments 17 e and 17 f, although dye was stored in the liver in excess of that found in the kidney, the amount stored in the liver was by no means as large as one might expect in cases in which the experiment had been so extended. The general impression one gets is that absorption by the mucosa is not a uniform and progressive process and that the ability of the mucosa to absorb the dye differs considerably among different individuals. This is well shown by a comparison of Experiment 16 d with Experiments 16 b and 16 c. In Experiment 16 d not a trace of dye was found in the epithelium of the kidney, while in Experiments 16 b and 16 c a few dye granules were observed (table 9). In cases where the number of feedings was considerable and the duration of the experiment extended, as in Experiment 17 g, the amount of dye stored in the liver and kidneys was so great, that no estimate can be definitely drawn between the amount relatively stored in each.

Mononuclear leucocytes containing dye granules were frequently met with in the intertubular tissue of the kidneys and occasionally in the liver. They were also often abundant in the mucosa of the alimentary canal. In a few instances they were found in the circulation (Exp. 17 f), and in some cases, as in sections of the kidney, they often appeared to be overloaded with dye and to be broken down (Exp. 17 f and 17 g). Mononuclears which had stored the dye were observed in the circulation of the pancreas (Exp. 16 b) and in the spleen (Exp. 16 b and 17 g).

The endothelium of the venules in the spleen did not appear consistently to take up the dye but in a few cases it appeared to be vitally stained (Exp. 17 g).

Without entering into further and unnecessary details, it may be stated that the reactions of typical tissues toward acid dyes, as far as the tissues themselves are concerned, do not appear to differ in the slightest degree in these experiments from those previously observed. In no instance was there any indication of nuclear staining or of a diffuse staining of the cytoplasm in the cells of the mucosa and submucosa in any of the individuals involved. In one instance, not included on table 9, the nuclei of the cells in the mucosa and submucosa, but not the cytoplasm, were found to be stained in a frog that had been dead for a few hours before fixation of the tissues. In this case, however, none of the other tissues appeared to be pathologic in the slightest degree. This frog had been fed sixteen times and died under treatment on the thirteenth day.

9. A COMPARISON OF THE REACTIONS OF THE TISSUES TOWARD COLLOIDAL ACID DYES AS OBSERVED IN EXPERIMENTS CONCERNED WITH THE PORTAL OF ENTRY OF THE DYE

On summarizing the above experiments concerning the portal of entry of the dye, we find in adult frogs and toads that solutions of colloidal acid dyes can gain admission to the body through the skin and intestinal mucosa. A striking difference is observed in the reactions of the tissues toward these dyes, however, in the two sets of experiments. When the portal of entry is restricted to the skin, dye is stored by the epithelium of the tubules in the kidney, before dye granules make their appearance in the stellate cells of the liver capillaries. On the other hand, when the portal of entry is restricted to the intestinal mucosa, the stellate cells of the liver capillaries store the dye before the epithelium of the kidney. Irrespective of its portal of entry, however, the mononuclear leucocytes of the body have often been observed to store the dye before any visible signs of dye can be found in the liver or kidneys.

When the portal of entry of the dye is restricted to the integument, the dye is taken up from the intercellular tissue spaces by the lymphatics, and conveyed with the transudated lymph to the venous circulation. The lymph and dye which enter the veins through the posterior lymph-hearts in the adult mixes with blood which is immediately and directly conveyed to the kidneys by the renal-portal (Jacobson's) veins. From the kidneys this blood is then conveyed by the postcava to the heart from which it is distributed to the pulmonary and cutaneous circulations. When lymph enters the venous circulation by way of the anterior lymph-hearts, it mixes with blood which, for the most part, must pass through the pulmonary and cutaneous circuits, before it can reach the liver and kidneys by the hepatic artery, portal vein, renal arteries or renal-portal veins. The circumstance that dye is first stored in the adult by the epithelium of the renal tubules, in cases where its portal of entry is restricted to the skin of the posterior regions, is probably due to the fact that the kidney is the first organ to be acted upon by the dye. In the case of young larvae in which the posterior lymph-hearts are wanting and the mesonephroi do not function, the pronephroi are without question reached by the dye solution before the stellate cells of the liver capillaries. The blood supply to the mesonephroi through the renal-portal veins in later larval stages is also more extensive and direct than that to the liver which may account for the earlier reaction of the mesonephroi in such cases.

If the passage of colloidal acid dye solutions through the intestinal mucosa is a process entirely analogous to that of certain intestinal fluids, dye might be taken up solely by the intestinal lymphatics and be conveyed to the venous circulation by the usual route. In which case the kidneys might even then be the first to store the dye. As dye solutions actually do pass through the intestinal mucosa, some dye probably reaches the venous circulation by this route. The circumstance that the liver is the first to receive and store the dye when absorption is restricted to the intestinal mucosa, and continues to store it in excess of that stored by the kidneys must, however, be explained in another manner.

Experiments thus far indicate that dye solutions of relatively low concentration do not pass through the intestinal mucosa of the adult. At least they do not appear to act on typical tissues like solutions of high concentration (Exp. 15, table 9). It is therefore highly probable in the case of solutions of high concentration, that the dye is absorbed not only by the mucosa and submucosa, but is taken up by the radicles of the portal vein as well. In this manner the dye would be able to reach the liver by a direct route where it becomes stored by the stellate cells which line the liver capillaries before reaching any other portion of the body.

Experiments in which the portal of entry is restricted to the integument show, in larval and adult frogs and toads, that the tissues react toward the dye in a uniform and progressive manner, both in relation to the amount of dye which enters the body, and to the length of time the animals are suspended in dye solutions. On the other hand, considerable variation was observed among different individuals in the ability of the intestinal mucosa to absorb the dye.

Feeding experiments on very young larvae have thus far proved impracticable. I am therefore unable to state in such cases to what extent, if any, the intestinal mucosa may absorb the dye. I am inclined to believe that the bluish appearance presented by the larvae in some cases, may be due to this cause. A few instances have been mentioned in the preceding experiments in which its absorption by the mucosa in some of the larvae may have possibly occurred.

10. ON THE REACTIONS OF THE TISSUES TOWARD COLLOIDAL ACID DYES DURING THE SEASON OF HIBERNATION

All of the experiments above-mentioned were conducted during the Spring and Summer months. It is therefore of interest to compare the reactions of the tissues toward colloidal acid dyes during what would ordinarily be the period of hibernation.

Large tadpoles of R. catesbiana obtained early in October and adult toads obtained in September were kept in the laboratory at ordinary room temperature up to the time the experiments were made. As the animals were kept in a fairly warm room and were more or less active during the Fall and Winter they have not, strictly speaking, been in a hibernating state. It may prove of interest, however, to compare the results of these experiments with those made during the Spring and Summer.

Experiment 18 (compare with table 8, p. 32). An adult toad was allowed to remain in a finger bowl containing a freshly-made 1:100 solution of Niagara blue for several hours daily, from December 7 to December 13, inclusive, and killed on December 14. The actual length of time the toad remained in dye during a period of seven days was thirteen hours and forty-five minutes.

The walls of the intestinal canal were slightly stained but the muscles of the body retained their natural color. A mass of dye was found in the rectum. The large abdominal lymph sacs were filled with an unusual amount of clear lymph which gave the body an oedematous appearance. Sections were made of the liver and kidneys.

A mere trace of dye was occasionally stored in some of the stellate cells of the liver capillaries. Not a trace of dye was found in the epithelium of the tubules in the kidneys. No mononuclears containing dye granules were observed in the intertubular tissue of the kidneys.

Experiment 19 (compare with Experiment 8, p. 22). *a*) A large tadpole of R. catesbiana with hindlegs was placed in a 1:1500 solution of trypan blue on November 20 and allowed to remain in the same until November 24, when it was killed (4 days in dye).

The merest trace of dye was found in a few of the stellate cells of the liver. Not a trace of dye was observed in the epithelium of the tubules in the kidney. No mononuclears containing dye granules were observed.

b) A large tadpole of R. catesbiana was placed in a freshly-made 1:1000 solution of Niagara blue on November 23 and remained in the same until November 28, when it was killed (5 days in dye).

Not a trace of dye was stored in any cell of the liver, kidney or spleen.

c) A large tadpole of R. catesbiana was placed in a freshly-made 1:1500 solution of Niagara blue on December 2 and remained in the same until December 8, when it was killed (6 days in dye).

Dye granules were stored in the epithelium of a number of tubules in the kidney and also to a much less extent in the stellate cells of the liver capillaries. Not a trace of dye was observed in the spleen. No mononuclears containing dye granules were found in the intertubular tissue of the kidneys. A number of large cells, however, probably mononuclear leucocytes, were found to be in an active state of division in the intertubular tissue of the kidneys. Similar cells were occasionally found in the liver and spleen. These cells were unstained.

Experiment 20 (compare with Experiment 12, p. 28). *a)* The tail of a large tadpole of R. catesbiana was suspended for short intervals daily from December 8 to December 11, inclusive, in a freshly-made 1:100 solution of trypan blue, and killed on December 13. The actual length of time the tadpole was suspended in dye during a period of five days was one hour and fifteen minutes.

The tadpole presented no external evidence of being stained by the dye except on the abdomen. This tadpole possessed two accessory spleens.

A mere trace of dye was stored in the stellate cells of the liver and a few dye granules were found in the epithelium of some of the tubules in the kidney. Not a trace of dye was observed in the spleen. No mononuclears containing dye granules were found in the intertubular tissue of the kidney. Large mononuclears in an active state of division, as observed in Experiment 19 c, were found in the intertubular tissue of the kidneys.

b) The tail of a large tadpole of R. catesbiana was suspended for short intervals daily from December 24 to December 28, inclusive, in a freshly-made 1:100 solution of Niagara blue and killed on December 29. The actual length of time the tadpole was suspended in dye during a period of five days was two hours and forty-five minutes. During this experiment the tadpole was kept in a cool room of which the temperature averaged about 10°C.

The merest trace of dye was stored in a very few of the stellate cells of the liver and only a very small amount was present in the epithelium of the kidney tubules. Not a trace of dye was observed in the spleen or in the mononuclears present in the intertubular tissue of the kidneys.

These experiments might indicate either that dye solutions do not gain admission to the body as readily during the season of hibernation as during the Spring and Summer, or, if they do, that the tissues do not as readily react toward the dye.

If we compare these experiments with essentially similar ones made during the Spring and Summer (Exp. 8 and 12) it is evident that the reactions of the tissues toward the dye during the season of hibernation are in general decidedly less pronounced. In only one instance was dye found to be really noticeably present in the liver and kidneys (Exp. 19 c). In two experiments its absorption wholly (Exp. 18 and 19 a) by the intestinal mucosa may have occurred. In both of the experiments (Exp. 20 a and 20 b) in which only the tail was immersed in the dye, the amount of dye stored in the kidney was more pronounced than that stored in the liver. Although these experiments clearly indicate that colloidal acid dye solutions do pass through the integument during the season of hibernation, there also appears to be a lack in the ability of certain recognized tissues to react toward the dye at this time. In other words, cells which ordinarily store the dye during the Spring and Summer appear to have become wholly or partially inactive toward the dye during the Winter months.

It is interesting to compare the behavior of advanced larvae of Diemyctylus viridescens toward solutions of colloidal acid dyes with those of Rana last mentioned.

Experiment 21. Three larvae of Diemyctylus measuring about forty-five millimeters in length were placed in solutions of trypan blue (1:1500, 1:2000 and 1:1000) on July 12 and were killed on July 16, 22 and 27, respectively, Not a trace of dye was observed in sections of the liver and mesonephros of those killed on July 16 (4 days in dye) and July 22 (10 days in dye), or in the mesonephros of the individual killed on July 27 (15 days in dye). In the liver capillaries of the latter, however, but not in the cytoplasm of the stellate cells, a bluish precipitate of the dye solution was observed.

If we may judge from the results of feeding experiments, abovementioned, this dye did not gain admission to the body through the integument, but reached the liver by way of the intestinal mucosa and portal vein.

11. ON THE CAUSES WHICH UNDERLIE THE INITIATION OF THE PROCESS BY WHICH DYE GRANULES ARE STORED IN THE CYTOPLASM OF CERTAIN TYPICAL CELLS OF THE EMBRYO

Experiments have shown us that the initiation of the process by which dye granules are stored in the cytoplasm of the cell is associated with the attainment of a distinctive ontogenetic larval stage. It is therefore of interest to inquire still further what these ontogenetic conditions actually may be.

When toad larvae are placed in solutions of colloidal acid dyes on the third and fourth days after fertilization, we have seen that visible signs of dye granules are not ordinarily found in the cell until the ninth day after fertilization. As some time always elapses when larvae are placed in solutions of colloidal acid dyes before dye granules appear in the cytoplasm of the cell, we must look for some stage of development prior to the ninth day at which the process of ingestion or absorption of dye particles is actually initiated.

It is evident that the storage of dye particles by the cytoplasm of the cell cannot possibly take place until the cell is brought into contact with the dye solution. When dye solutions gain entrance to the body through the integument it would therefore be essential, in order for the dye to reach the typical cells at such a time, for circulatory conditions to be favorable for the transference of dye from the intercellular tissue spaces to the general circulation. We should therefore examine the circulatory system of the earlier larval stages to determine when such conditions might first be found. The earliest stage of development in the toad which would permit of the transference of lymph by the lymphatics from the periphery to the veins, has been found to occur, approximately, between the fifth and sixth days after fertilization. We may therefore consider in some detail the general ontogenetic conditions which prevail at this time.

On the fifth day after fertilization, the ventral and lateral superficial lymph sinuses of the head are represented in Bufo by a number of *independent and disconnected anlages or endothelial-lined vesicles or sacs*. These anlages neither communicate with each other, with the veins nor with any other lymphatics at this time. This particular stage of their development has been well illustrated by Kampmeier in the toad ('15, fig. 12). The anterior lymph-hearts are present and are situated just caudal to the pronephros. These lymph-hearts are often filled with blood-corpuscles and, *as no valves are present*, they are in wide-open communication with the veins. A lymphatic opens dorsally into the lymph-heart which can be traced craniad for only a short distance at this stage. This lymphatic also often contains blood-corpuscles as no valves between it and the lymph-heart have been formed. It has been designated by Hoyer ('08) as the ductus cephalicus and corresponds to the lateral pharyngeal lymphatic in the embryo of the trout (McClure, '15).

On the fifth day after fertilization no lymph can possibly reach the anterior lymph-hearts or general circulation from the lymph sinuses of the head region, because the sinuses have not established a connection with the lateral pharngeal lymphatic (ductus cephalicus) and do not otherwise communicate with the veins. No blood corpuscles of any character were found in the cephalic lymph sinuses on the fifth day, which is also significant of the fact that lymph or blood does not circulate through them at this time.

A blood-vascular circuit is in active operation through heart, arteries, capillaries and veins. All of the blood from the posterior regions is returned to the heart by the postcardinal veins. The proximal or hepatic portion of the future postcava lies in direct contact at the hepatico-subcardinal junction with the right subcardinal vein (McClure, '06, p. 168). As a communication has not been established between these two veins at this juncture, *a postcaval circulation has not yet been formed.* The external gills are highly developed and their capillary circulation active. Small loops of blood-vessels extend for a short distance into the caudal fins. No lymphatics, however, are found in the fins.

The thyroid is detached from the floor of the pharynx but is not yet completely separated into a right and left gland. A number of pronephric tubules are present but only two nephrostomes for each kidney have been formed. The right mesonephros is represented by three small solid anlages but no hollow renal vesicles have been formed. The anlages of the left mesonephros are much smaller and less distinct than those on the right side.

White blood-corpuscles are met with in various stages of differentiation in the general blood-vascular circulation, pronephros and mesenchyme. Many of them are in an active state of division and some of them are approaching the typical mononuclear form. The general impression one obtains is that none of these cells have completed their differentiation and that none of them could now be regarded as typical leucocytes. No indication of any cells arising from lymphatic endothelium was observed at this stage.

The distinctive features observed on the fifth day, are the disconnected character of the anlages of the lymphatics in the head region and the undifferentiated character of the white blood-cells. It is also to be noted that one of the main blood-vascular channels has not been established, as the postcava is not yet formed.

On the sixth day after fertilization in Bufo marked changes in the organs of circulation are observed. The original and early independent anlages of the ventral cephalic lymph sinus are no longer found. *These anlages have now become confluent to form a large single-chambered sinus* which occupies the position of the submaxillary lymph sinus in the adult. This lymph sinus of the embryo is superficially located beneath the integument and occupies an extensive area in the territory ventral to the mouth (see Kampmeier, '15, Plate 1). It communicates on each side of the head with the lateral cephalic lymph sinus which is also superficially located, and through which it now drains into the lateral pharyngeal lymphatic (ductus cephalicus) and thence on through the anterior lymph-hearts into the veins.

The above observations leave no room for doubt that a continuous lymph flow is now established for the first time between the large superficial lymph sinuses of the head and the veins. From the conditions observed on the fifth day, this drainage of the head sinuses into the veins must have been initiated in Bufo sometime between the fifth and sixth days.

The lymph from the posterior regions now drains into the anterior lymph-hearts by superficially located lymphatics. One of these, the lymphatic of the lateral line, as in the trout embryo (McClure, '15), forms the direct caudal continuation of the lateral pharyngeal lymphatic (ductus cephalicus). *Definite valves are now present for the first time between the anterior lymph-hearts and the veins and between the anterior lymph-hearts and the lateral pharyngeal lymphatic.*

A few blood-corpuscles were often found in the anterior lymph-hearts which entered from the veins before valves were formed. A similar condition has been observed by the writer ('15, p. 11) in the embryo of the trout.

Coagulated lymph and an occasional typical mononuclear leucocyte are found in the large lymph sinuses of the head. Mononuclears are also found occasionally in the pronephros and mesenchyme. Many free cells in the mesenchyme, circulation and pronephros are, however, still in an active state of division. It is only essential to mention here in this connection, that blood-cell development is more advanced than on the fifth day, and that some of the free cells resemble what one would regard as typical mononuclear leucocytes. It would appear that the presence of a few typical leucocytes at this time, coincides with the opportunity presented to them of ingesting waste products of metabolism, which may now be brought to them by the lymphatics from the intercellular tissue spaces.

A communication has been established at the hepatico-subcardinal junction between the liver circulation and the right subcardinal vein, so that the *postcava is now definitely formed.* This communication is quite insignificant, however, and most of the blood from the posterior regions still passes to the heart by the postcardinal veins.

The thyroid glands of the right and left sides are still connected posteriorily. The tubules of the pronephros have increased in number and three nephrostomes are formed. This is the same number of nephrostomes found in Bufo on the ninth day after fertilization. The right mesonephros is represented by six renal vesicles and the left by five. The vesicles of the right side are larger than those of the left. Some of the former are hollow but none of them connect with the pronephric duct.

The external gills are still highly developed and their capillary circulation is in full swing. The blood-vascular loops of the tail now reach about half-way into the caudal fin. No lymphatics are present in the caudal fin. *In none of the larvae was dye observed in the intestinal canal.*

The external gills begin to atrophy in Bufo between the sixth and seventh days after fertilization and are completely lost in most cases at the beginning of the seventh day. As observed by Hoyer ('08), one can then more readily observe the rhythmical contractions of the anterior lymph-hearts. Hoyer has stated that the formation of valves in the lymph-heart coincides in the frog with the loss of the external gills. In B. lentiginosus the external gills are still present when the valves are formed sometime between the fifth and sixth days.

From the sixth day on up to the ninth day after fertilization, when direct evidence of phagocytosis may ordinarily be first observed in certain cells, there is naturally a gradual and progressive increase in the amount of lymph which passes from the periphery to the veins.

The above observations decidedly indicate that a continuous lymph flow from the intercellular tissue spaces of the head region to the venous circulation, is inititated in the toad embryo sometime between the fifth and sixth days after fertilization. Prior to this time neither the transudated lymph nor the dye solution which must first enter the tissue spaces, can possibly reach the general systemic circulation by the lymphatics. When a continuous lymph flow is established between the periphery and the veins, the dye which is mixed with the lymph is then able to reach all of the tissues by which it is ordinarily stored. *In other words, the initiation of the process of ingestion of dye particles by the macrophages and of their absorption by renal epithelium, appears to be coincident with the establishment of a continuous lymph flow through the lymphatics between the periphery and the veins.*

The dye solution does not appear to gain admission to the tissue spaces of the embryo until a continuous lymph flow is established between the periphery and the veins. If it did, it would mix with the transudated lymph in the tissue spaces and might enter the independent anlages of the head sinuses, or even the anterior lymph-hearts which, even on the fourth day, are in wide open communication with the veins. Prior to the establishment of a continuous lymph flow from the periphery to the veins, no dye has thus far been observed in any of the lymphatic anlages or in any tissue of the body.

The amount of dye which reaches the venous circulation by the lymphatics from the tissue spaces, must be directly related to the available lymphatic pathways developed at a given time. When a continuous lymph flow from the periphery is first established in the embryo the amount of dye which reaches the general circulation must be relatively small and its action on the tissues correspondingly slight. As the amount of dye which reaches the venous circulation necessarily increases progressively with the development of available pathways, its ingestion or absorption by the typical cells, as the case may be, is gradually hastened up to the time all of the lymphatics are formed. At such a time, as in the case of advanced larvae, the storage of dye granules may then even be accomplished in some cases within twenty-four hours (Exp. 6, 7, 11 and 12).

While under the conditions of the above experiments (Exp. 1, 2 and 3) dye granules ordinarily make their first appearance in the cytoplasm of the cell on the ninth day after fertilization, their deposition might possibly be somewhat hastened, if dye solutions of higher concentration were used. (Compare Exp. 10 c and 11). In any event, however, dye could not reach the general circulation from the tissue spaces of the head region by the lymphatics until the cephalic lymph sinuses had established their connection with the veins.

The above observations seem to confirm a recent observation of the writer concerning the establishment of a continuous lymph flow between the tissue spaces and veins in the embryo of the trout (McClure, '15a).

It was found prior to about the twenty-second day after fertilization in the rainbow and steelhead trout, that the lymphatics of the head and pharynx are represented by a discontinuous system of independent and disconnected anlages which consist largely of endothelial-lined vesicles or sacs. Two of these independent anlages, the subocular lymph sacs, are relatively huge structures situated ventral to the eyes which can be observed, injected or otherwise experimented on in the living embryo. During their independent stage these sacs collect transudated lymph from the tissue spaces and store it temporarily until they establish a connection with the lateral pharyngeal lymphatic, through which they then drain into the veins. At this time also valves begin to be formed between the lymphatics and the veins (McClure, '15).

It will be observed that a continuous lymph flow between the head sinuses and the veins is initiated in the same manner in the embryo of the toad and the trout, by a confluence between the independent head sinuses and the lateral pharyngeal lymphatic (ductus cephalicus of Hoyer). Whether the head sinuses in Bufo also collect transudated lymph from the tissue spaces, prior to the time they drain into the veins, has not been definitely determined up to the present time.

It might be possible by means of subcutaneous injections of dye solutions into trout and chick embryos, to confirm the above observations made on the toad. If the principle outlined above for the toad is correct, one would expect it to be so in all cases.

Intravenous injections of embryos at a time prior to the establishment of a lymph flow between the periphery and veins, might also lead to interesting observations regarding the functional state of the renal epithelium, as well as that of other tissues at such a time. Such experiments would be analogous to cases in which dye might be absorbed by the intestinal mucosa before the lymph flow was established between the periphery and the veins.

On the fifth day the intestinal canal of the toad larva is filled with yolk which would make it impossible for dye to pass through. No dye was observed in the intestinal canal of the living larvae until the seventh day, although conditions were such that it would have been possible for dye to pass through sometime between the fifth and sixth days. At the time when a continuous lymph flow is first established through the lymphatics between the tissue spaces and the veins, it is therefore not impossible that dye may at the same time gain admission to the body through the intestinal mucosa or may be taken up directly by the veins (Moore, '15). Irrespective of the possibility that dye may also reach the general circulation by a route other than by the lymphatics, it is clearly evident that it does not do so until a continuous lymph flow is established through the lymphatics between the tissue spaces and the veins.

12. DOES THE METHOD OF VITAL STAINING BY COLLOIDAL ACID DYES CONTRIBUTE ANY EVIDENCE IN FAVOR OF THE VIEW THAT THE LYMPHATICS ARE DERIVED FROM THE VEINS?

Regarding the view that lymphatics are derived from the endothelium of the veins, Wislocki ('16 a, p. 127) states as follows:

> Three views have assumed the most important places among the numbers of theories which have been evolved to explain the development of new lymphatic vessels.
>
> 1. That lymphatics may result from a transformation of blood-vessels, and that extension of the lymphatic system results from the coupling on or addition of these changed blood-channels.
>
> 2. That the endothelial cells of the lymphatics may arise from cells of the extra-vascular mesenchyme.
>
> 3. That after the initial outgrowth of lymphatics from the veins, in the form of lymph-hearts has occurred, neither mesenchyme cell nor blood-vessel endothelium contributes any further to their growth; but

> that they are an independent tissue, growing by division of primary lymphatic endothelium, and invading the embryonic body, as a vine climbs a trellis.
>
> These three views, as they apply to the tadpole, can be subjected to a *crucial test*[4] in the light of the discovery that trypan-blue is an elective stain for amphibian embryonic lymphatic endothelium. Experiments necessary to this end are being prosecuted in this laboratory. Even at the present time, while these experiments with vital dyes are only in their initial stages, it is possible by their help to draw certain fundamental conclusions, which may aid in directing the study of lymphatic growth.
>
> The view maintained by a second group of writers, namely, that lymphatics grow by the[5] . . . . transformation of mesenchyme into endothelium, clearly becomes untenable, since mesenchyme and lymphatic endothelial cells are shown by their reaction toward the dye to be biologically different; and conversion of one into the other becomes extremely improbable.
>
> The observations made so far upon vitally stained larvae, substantiate and add additional proof to the interesting observations of E. R. Clark on the growth of lymphatics in the tadpole's tail. The author is able to confirm Clark's view that these lymphatics grow only by sprouting, and by sending out fine protoplasmic processes, which gradually become definite lumen-containing sprouts.

Let us now see to what extent Wislocki's method of vitally staining the lymphatics really contributes evidence, as he claims, that the lymphatics of the frog are derived from the endothelium of the veins.

Evidence from the standpoint of vital staining by colloidal acid dyes that the lymphatics grow out from the veins, would at least call for the demonstration of an early stage of growth, at which a vitally stained lymphatic is in the process of growing out from a vein. I may state that not even the faintest approach to such a demonstration can possibly be realized either in the embryo of the frog or toad. The reason for this is plain. The lymphatic system of the frog and toad does not become vitally stained, so to speak, by colloidal acid dyes, until an extensive system of continuous lymphatic vessels have been formed, which convey lymph from practically all regions of the body to the anterior lymph-hearts and thence to the veins. It is also significant to note that the endothelium of the anterior lymph-hearts in the frog and toad from which, by some investigators, the lymphatics are supposed to grow, is not vitally stained by colloidal acid dyes. As a matter of fact, the earliest stage of development at which the lymphatics react toward these dyes in the frog and toad, is one not very far removed from their permanent larval form. Such an advanced stage would not ordinarily be regarded as a favorable one from which to infer in what manner the lymphatics have been formed.

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[4] Italics added by the writer.

[5] The portion omitted from the quotation refers to extraintimal and perineural lymphatic spaces which thus far have not been observed in the frog or toad. (McClure, '15, p. 66.)

The method of vital staining by colloidal acid dyes, like the injection method as ordinarily used, does not therefore demonstrate the existence of lymphatics in the embryonic body, until their development is far advanced and until after continuous channels are formed. This method, also like the injection method, is incapable of demonstrating the early independent anlages of the lymphatics, like those of the head sinuses in the frog and toad, which at one time are entirely independent of each other and of the veins (Chapter 11).

The 'crucial test' referred to by Wislocki of the first and second 'views' therefore completely fails, as a vital staining of the lymphatic tissues by his method is not possible at a time when such conditions might arise. It would not be impossible, however, by this method to follow the growth of lymphatics which may sprout from other lymphatics. This would not prove, however, that such lymphatics had grown out continuously and centrifugally from the veins.

So much stress has been laid to the circumstance that lymphatics grow into the caudal fin of the tadpole's tail, it may not be out of place to discuss here more fully the real significance of this observation.

It must be borne in mind that Clark's ('09, '12) observations are confined exclusively to the growth of lymphatic endothelium in the caudal fin and that *this endothelium does not grow into the fin from the endothelium of a vein, but from that of another lymphatic.*

The circumstance that lymphatics grow into the tadpole's fin from other lymphatics, has been sufficient proof, in the minds of some, that all other lymphatics of the body must also originate by growth and that their source of origin must of necessity be the endothelium of the veins. The circumstance that lymphatics have actually been observed to sprout and grow from other lymphatics constitutes, therefore, the chief evidence upon which the theory of the venous origin of the lymphatics rests.

The adherents of this view might similarly infer from the growth of blood-vessels in the fin of the tadpole's tail, or elsewhere, that all other blood-vessels in the body must also originate by growth from some pre-existing endothelium, whose source of origin must of necessity be the unit vascular anlage designated by His as the 'angioblast.' As a matter of fact, this is exactly the line of reasoning followed by them. The assumption of a unit vascular anlage as a source of all vascular endothelium, both haemal and lymphatic, is the only logical conclusion of such a theory of general endothelial growth.

Recent experiments[6] have shown, however, that a unit vascular anlage, the angioblast, as a source of all endothelium, is a myth and does not exist. What has been found to exist is that endothelium arises discontinuously *in situ* from mesenchyme, throughout the embryo, and in the exact location in which recognized vessels appear. The assumption, therefore, upon which the theory of a continuous growth of endothelium throughout the embryo is founded, has been proved by experiment to be incorrect.

How can we reconcile the fact that intraembryonic endothelium does arise *in situ* from mesenchyme with the fact that it has also been observed to sprout and grow? The answer to this question by adherents of the continuous growth theory should of necessity be a flat denial that such a condition could conceivably exist. We now know, however, that such a condition actually does exist, and there must be some plausible explanation for it.

As a matter of fact it would be inconceivable to imagine endothelium incapable of growth. How could we otherwise explain the growth of the vascular system from the embryonic to the adult stage? We can reconcile the fact of a local differentiation of mesenchyme into endothelium with the latter's ability to grow, on the recognized grounds that endothelium, after its differentiation from mesenchyme is completed, forms no exception to other differentiated tissues in its general ability to grow. As endothelium naturally cannot grow, as such, until it has been formed, its local genesis from mesenchyme must be regarded as a process distinct from that of its subsequent growth. Both processes combined, however, constitute its development.

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[6] The reader is referred to the works of McClure, '15, '16 and Reagan, '17 for the literature relating to these experiments.

The theory of the local origin of endothelium from mesenchyme was originally based on purely morphological observations and not on experiment. Now that experiment has confirmed this view we are doubly assured of the accuracy of our morphological observations, as well as of their interpretations. The identical morphological observations so uniformly made for the local origin from mesenchyme of lymphatic and blood-vascular channels, can therefore leave little room for doubt, as regards a corresponding identity in their mode of development.

As previously stated by the writer ('15, '15b):

> The development of the general vascular system, both haemal and lymphatic, is a uniform process which consists in a local origin (genesis) of endothelium from mesenchyme, and a growth of endothelium after it has once been formed.

13. THE PROGRESSIVE REACTIONS OF THE TISSUES TOWARD COLLOIDAL ACID DYES AS OBSERVED IN THE COURSE OF LARVAL DEVELOPMENT

The progressive reactions of the tissues toward colloidal acid dyes, as observed in the course of larval development, is a question of interest and importance. If these reactions are indicative of the functional activity of the tissues, one should be able to determine approximately when certain organs first begin to function in the embryo. In addition to the pronephros, as observed at the critical ontogenetic stage (p. 13), the organs which lend themselves especially to such an investigation are the mesonephroi. The spleen, thymus gland, bone-marrow and the different types of white blood-corpulses might also possibly be studied in this manner.

In studying the reactions of tissues toward acid dyes, as observed at any particular stage of development, it should be borne in mind that the extent to which such reactions may occur at any time, is not only influenced by the length of the immersion of larvae in dye solutions, but can also be modified by varying the experiment. A rather prolonged treatment with dye is therefore essential, in order to be sure that all of the cells have stored the dye which are potentially capable of doing so at a given stage of development.

The mesonephroi. The anlages of the mesonephroi were observed to make their first appearance in larvae of Bufo sometime between the fourth and fifth days after fertilization. It is unnecessary to state that the time indicated for this or for the occurrence of any other event mentioned in this paper is purely a relative matter. The rate of development varies slightly in different individuals, and may even be modified experimentally by a number of conditions.

The right mesonephros is from the first more highly developed than that of the left side and remains so until a relatively late stage of development. Even as late as the sixty-eighth day after fertilization considerable difference in size still exists between the organs of opposite sides. What the cause of this asymmetrical plan of development in Bufo may be I am unable to determine. A loop of the intestine presses closely against the ventral surface of the left mesonephros. This does not seem sufficient, however, to account for the asymmetry mentioned.

The presence of dye granules in the epithelium of the tubules in the right mesonephros was first observed in a few individuals killed on the tenth day after fertilization. The toad larvae on which these observations were made were placed in the dye solution on the third (table 1) and fourth (table 2) days after fertilization and had therefore experienced a long and continuous immersion. In all cases the dye granules were exceedingly small and few in number and appeared to be confined to the epithelium of a single tubule. From the tenth day on the granules increased in number and on the thirteenth day (table 1) they could be identified in all of the larvae even with a fairly low power objective. At no time up to the fifteenth day could the right mesonephros be regarded as a very active organ, as the absorption of dye was still being carried on almost entirely by the epithelium of both pronephroi. The epithelium of the pronephros which has stored the dye has by this time lost all semblance to its original character and it is remarkable that, under such extreme conditions, absorption should also be confined exclusively to so few tubules. In a few cases the excess of dye which could not be cared for by the epithelium of the right pronephros was found to have stained the epithelium of the Wolffian duct quite deeply and diffusely.

Although the first indication of the absorption of dye by the epithelium of the right mesonephros can be observed in Bufo on the tenth day after fertilization, the renal epithelium does not react toward the dye at this time unless the larvae have previously experienced a rather long immersion. When toad larvae were placed in a solution of dye on the ninth day after fertilization (table 5) no indication of the presence of dye granules was observed in the epithelium of the right mesonephros until the thirteenth day. In toad larvae placed in a 1:1500 solution of trypan blue on the eleventh day after fertilization, dye granules also made their first appearance in the epithelium of the right mesonephros on the thirteenth day. In this case as in all of those previously mentioned, the dye granules were exceedingly few in number and appeared to be confined to a single tubule.

The first appearance of dye granules in the epithelium of the left mesonephros was not observed until the fifteenth day in the series of toad larvae which had experienced a long immersion in dye (table 1). In a series of toad larvae placed in a solution of dye on the eleventh day after fertilization dye granules were also first observed in the epithelium of the left mesonephros on the fifteenth day.

It is interesting to note when toad larvae were placed in a dye solution on the ninth day after fertilization (table 5, critical ontogenetic stage), that the mesonephros of the left side did not appear to be much further developed on the twentieth day, than at the time the larvae were first placed in the dye. Up to this time, no dye granules had been stored in the epithelium.

In comparing the above observations with conditions which might normally occur, we must take into consideration the unusual environment in which development has taken place. Be this as it may, we are able to obtain an approximate estimate of the time at which the renal organs first begin to function in the capacity of eliminating waste material from the circulation. We can therefore state that dye granules may make their first appearance in the pronephros of toad larvae on the ninth day (p. 13), in the right mesonephros on the tenth day and in the left mesonephros on the fifteenth day after fertilization. The presence of dye granules would at least indicate that the functional activity of these organs has only quite recently been established.

One of the most striking features observed in a study of adult and larval tissues, is the apparent inequality in the ability of cells of exactly the same type to store the dye. Attention has already been drawn to this by Hektoen ('11), Rosenow ('06) and others.

It is not unusual in toad larvae to find an endothelial cell of the lymphatic or venous systems which is loaded with dye granules to be lying contiguous to another cell of the same type in which not a trace of dye is present. Corresponding observations can be made for the stellate cells of the liver capillaries. Equally striking is the presence of free cells loaded with dye granules which lie in the tissues side by side with exactly similar types of free cells in which no dye granules are present. At the same time, as has been so uniformly observed by all investigators, white blood-corpuscles containing dye granules are rarely found in the circulating blood and not to any extent, even after larvae have experienced a prolonged immersion in dye. The explanation of this latter phenomenon has, as we know, been one of the mooted questions. Without pretending to furnish any final solution to the problem a few observations recently made by the writer may be of interest in this connection.

Downey ('17a) has recently shown that cells which do not ordinarily take up the dye in the circulation, as polymorphonuclears and lymphocytes, may be made to do so by isolating them in a segment of the femoral vein which has been doubly ligatured and into which, between the ligatures, a 1 per cent solution of pyrrhol blue has been injected. Downey (p. 443) states:

> The next day the isolated segment was removed, fixed in Helly's Zenker-formol mixture, imbedded and sectioned. . . . . All of the polymorphonuclears which came in contact with the dye are loaded with it, and it is concentrated in the form of the typical dye granules. There is no diffuse staining of cytoplasm or nucleus, which indicates that the leucocytes were living at the time of fixation.

Downey's observations are most suggestive and indicate that cells which do not ordinarily take up dye while in the circulation may be made to do so in a typical manner when placed in a modified environment. The fact that these cells store the dye in a typical manner, certainly shows that their inability to do so while in the circulation is not necessarily due to the incompleteness of their differentiation.

Although I believe Downey may have settled a very important feature of the general problem by showing, on the basis of vital staining, that such free cells cannot be classed apart from those found in the tissues, we are still in doubt why some free and fixed cells react toward the dye, while other contiguous cells of exactly the same type often behave differently under similar environmental conditions. While environment may finally play an essential rôle in causing the free cells in the circulation to manifest their activities, after entering the tissues, neither it nor an incomplete state of differentiation of the cells can wholly account for the above-mentioned variability observed in larval and adult animals.

Experiments (Chapter 10) seem to show that certain typical tissues of adult frogs and toads and of well-advanced larvae do not react at all or do not as readily react toward the dye during the Winter months, as during the Spring and Summer. It has also been shown in these experiments that this is not due entirely to the circumstance that these tissues may not have been sufficiently acted upon by the dye. The circumstance that cells which have previously been capable of storing the dye in the Summer are unable to do so during the Winter, cannot be explained on the basis of the incompleteness of their differentiation, but rather on the basis that they have become temporarily inactive toward the dye. We also know that these same cells would again actively react toward the dye in the following Spring. We therefore appear to possess evidence that certain typical cells of the body may normally undergo temporary and alternating phases of activity and inactivity, as regards their reactions toward colloidal acid dyes.

It has also been observed by the writer that the reactions of lymphatic endothelium toward colloidal acid dyes are much more pronounced during early larval stages, than in young frogs with rudimentary tails and in which both pairs of limbs are free and fully formed. When a young frog of this character has remained in a 1:1500 solution of dye No. 161 for seventy hours and after this for twenty-four hours in tap water before killing and fixation, the endothelium of the large superficial lymph sinuses of the head and posterior regions does not store the dye as actively as in young larvae within the time limits, at least, of this experiment. The endothelium of the subvertebral lymph sinuses and some of its tributaries, however, is intensely phagocytic; also the endothelium of certain other smaller lymphatics found here and there throughout the body. It is evident that large areas of lymphatic endothelium which were actively phagocytic during early larval stages have ceased to be so at a more advanced age.

These observations on hibernating animals and young frogs suggest the possibility that the above-mentioned variability, as regards the reactions of typical tissues toward acid dyes, may also be explained on the grounds of the temporary and alternating states of activity and inactivity which such free and fixed cells may undergo.

In relation to recognized regions of the pharynx where dye is absorbed by the epithelium, the distribution of the mononuclear leucocytes in the tissues of the above-mentioned young frogs is most significant. These regions, as stated on a preceding page (p. 13), are thickened patches of epithelium which occupy a position in both the frog and toad in the roof and floor of the pharynx. Great numbers of mononuclear leucocytes loaded with dye granules are found in the connective tissue contiguous to these two regions of the pharynx. These leucocytes are even found in the epithelial layer itself where it is not unlikely that they actually ingest the dye particles previously stored by the epithelial cells of the pharynx.

14. SUMMARY

1. Among 140 larvae of Bufo which were placed on the third and fourth days after fertilization in solutions of colloidal acid dyes (Niagara and trypan blue), it was found that dye was stored in certain typical tissues of only 3 of the 66 larvae killed at successive intervals previous to the ninth day. In all except 2 of the 31 larvae killed on the ninth day, and in all of the 43 larvae killed at successive intervals subsequent to this time, dye was stored in the form of dye granules in the cytoplasm of certain typical cells.
2. Essentially similar results were obtained with frog larvae.
3. These experiments indicate that the initiation of the process by which colloidal acid dye particles are stored in the cytoplasm of the cell, must be associated with the attainment of some distinctive ontogenetic larval stage, rather than with the length of time larvae are acted upon by dyes.
4. The initiation of the process of ingestion of colloidal acid dyes particles by the macrophages and of their absorption by renal epithelium, appears to be coincident with the establishment in the embryo of a continuous lymph flow through the lymphatics, between the tissue spaces and the veins.
5. After the critical ontogenetic stage has been passed, the storage of colloidal dye particles by typical tissues may be accomplished within a relatively short time in both larval and adult frogs and toads.
6. Experiments on larval and adult frogs and toads show that colloidal acid dye solutions can gain entrance to the body through the integument. Feeding experiments on adults also show that colloidal acid dye solutions may be absorbed by the intestinal mucosa and reach the general circulation by the portal vein.
7. During the Winter the reactions of the tissues in adult frogs and toads toward colloidal acid dyes appear to be much less pronounced than in the Spring and Summer. Immersion experi-

ments seem to show that tissues which ordinarily react in Summer, may not readily react, or may not react at all toward the dye during the Winter months.

8. The method of vital staining by colloidal acid dyes presents no evidence whatever in favor of the view, as claimed by Wislocki, that the lymphatics of the frog are derived from the endothelium of the veins. Lymphatic endothelium does not react toward these dyes in the frog and toad until practically all of the main lymphatic channels have been formed and have begun to function in returning lymph from the tissue spaces to the veins. Such an advanced stage of development would not be ordinarily regarded as a favorable one from which to infer in what manner the lymphatics may have been formed.

9. Mononuclear leucocytes with eccentrically situated nuclei, appear to be the chief free macrophages found in the tissues of anura which most readily react toward colloidal acid dyes.

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